Amendments and Corrections

(1) Section 1.2 (b), pages 4-5
Duplicated paragraph removed.

(2) Figure 1.1, page 8
Inserted text:
"Molecules involved in the transitions between the subsets are shown at the relevant points, where $x^{-/-}$ means the phenotype observed in the knockout of molecule *x*."

(3) Section 1.2 (c), Negative Selection, page 11
Inserted "to" in paragraph 5.
"In a similar way, the peritoneal cavity microenvironment of anti-erythrocyte transgenic mice allows auto-reactive B-1 cells **to** accumulate (Murakami, 1992)..."

(4) Section 1.4(b), Memory B cell phenotypes and locations, page 34
Removed "#44" from reference list in paragraph 2.

(5) Section 2.12(a), Cytoskeletal Stabilising Buffer (CSB) Lysis, page 72
Inserted text, paragraph 1:
"CSB buffer components are described in Section 2.13."

(6) Section 2.13, Stock Solutions, page 76
Added CSB lysis buffer recipe.

(7) Figure 3.4, Part A, page 90
Deleted text: "IgM and IgD (MD-4)"
Now reads:
"ELISPOT data from day 5 after transfer and immunisation demonstrating the equivalent behaviour of transgenic lines expressing the IgG membrane tail (MG2, MG6 and MG8) compared to lines expressing IgM alone (MM-4) or IgMδE (MδE1 and MδE2)."

(8) Figure 6.2, page 158
Inserted text. Now reads:
"The results are given as the number of anti-lysozyme IgG_1 AFCs **(closed circles)** and the number of anti-lysozyme IgM^a AFCs **(open circles)** per 10^7 recipient splenocytes."

(9) Section 6.3(c), IgG, IgM/G and IgM transgenic lymph node B cells retain differential responses to antigen, page 161, paragraph 2
Deleted text, paragraph 2. Now reads:
"Mature lymph node B cells from IgG:$RAG1^{-/-}$, IgM/G:$RAG1^{-/-}$ and IgM:$RAG1^{-/-}$ mice were transferred along with TCR transgenic T cells into immunised recipients."

Stephen W. Martin, 26th July, 2002

B cell antigen receptor isotypes: implications for immune memory

Stephen W. Martin

December, 2001

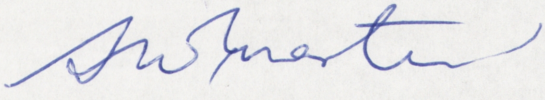
A thesis submitted for the degree of Doctor of Philosophy
of The Australian National University

Medical Sciences Graduate Program, John Curtin School of Medical Research,

Australian National University, Canberra

This thesis presents research undertaken at the Medical Genome Centre, Division of Molecular Medicine of the John Curtin School of Medical Research, Australian National University, Canberra. This work was performed between July 1998 and December 2001, while I was the recipient of an Australian National University PhD scholarship.

This is to certify that the studies outlined in this thesis are my own work, performed under the supervision of Professor C. Goodnow. Valuable assistance was also provided from other members of the Goodnow lab, the staff of the ACRF Medical Genome Centre and the staff of the John Curtin School of Medical Research, Australian National University, Canberra, as stated in the acknowledgments. This thesis is under 100,000 words.



Stephen W. Martin, December 2001

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Above all my sincere thanks to my supervisor and mentor Professor Chris Goodnow for his vision, clarity of thought and boundless positive energy which has given direction to this study. Chris is a remarkable scientist and has been an approachable, patient teacher.

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Finally, I would especially like to thank my family, Mum, Dad and Jen, and my wonderful partner and best friend Emma-Kate for their continual love, support and encouragement. Thanks for being there for me.

A human being is part of a whole, called by us the "Universe", a part limited in time and space. He experiences himself, his thoughts and feelings, as something separated from the rest - a kind of optical delusion of his consciousness. This delusion is a kind of prison for us, restricting us to our personal desires and to affection for a few persons nearest us. Our task must be to free ourselves from this prison by widening our circles of compassion to embrace all living creatures and the whole of nature in its beauty.

- Albert Einstein

Abstract

The B cell antigen receptor (BCR) can be expressed in a number of molecular forms during B cell development, and provides context-dependent signals that lead to a variety of cellular outcomes. Isotype switching during an immune response represents a key, irreversible molecular change in BCR structure, where naïve BCR isotypes (IgM and IgD) are replaced by switched isotypes (IgG, IgA, IgE) that become markers of antigen experience. Attempts to study isotype-specific BCR signalling *in vivo* have been hampered, however, by the changes in BCR affinity, location, cell surface phenotype, activation requirements and lifespan that also accompany antigen priming.

To address the role of isotype-switched BCR expression during an immune response, homogeneous populations of naïve B cells derived from anti-lysozyme immunoglobulin transgenic mice were seeded into a T-dependent immune response to lysozyme *in vivo*. Comparisons were made between transgenic B cells expressing IgM and IgD BCRs and B cells expressing full length IgG or the unique IgG membrane tail domain.

It was found that naïve transgenic B cells expressing the IgG membrane tail domain make a more robust response to antigen compared to naïve transgenic B cells bearing IgM, both in terms of net clonal expansion and in the production of antibody-forming cells. Transgenic B cells expressing the IgG membrane tail were not activated more efficiently, nor did they have a greater rate of cell division compared to IgM transgenic B cells. Rather, the IgG membrane tail protected B cells from cell death during the process of clonal expansion.

This experimental strategy was verified by showing that B cell developmental differences in the bone marrow, as well as phenotypic variations in the marginal zone and follicular B cell subsets of the various transgenic lines did not account for their responses to antigen *in vivo*. The only apparent determinant of reactivity to antigen in this system was BCR isotype and the expression of the IgG membrane tail domain.

This study has identified BCR isotype as a regulator of B cell responses to antigen during an immune response *in vivo*. It also shows that the IgG membrane tail is the key molecular determinant of the protective effect of IgG expression, leading to increased cell survival during the process of clonal expansion. Isotype switching to IgG may therefore represent a critical component of B cell priming, allowing greater magnitude expansion of memory B cells during a memory response.

Publications

Arising from this work:

Martin, S. W. and Goodnow, C. C. (2001) Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory, *Nature Immunol.*, accepted for publication.

Selected conference papers:

Martin, S. W. and Goodnow, C. C. (2001) The IgG_1 cytoplasmic tail domain is an important regulator of B cell clonal expansion, Keystone Symposium: *"B cell Immunobiology and Disease"*, Snowbird Resort, Utah, U.S.A.

Martin, S. W. and Goodnow, C. C. (1999) BCR isotype can influence terminal B cell differentiation: implications for B cell memory, 29th Annual Conference of the Australasian Society for Immunololgy, Dunedin, New Zealand

Other publications:

Martin, S. W. and Goodnow, C. C. (2000) Memory needs no reminders (News and Views), *Nature* **407**, 576-577 (re-printed with permission as an appendix)

Contents

Figures and Tables

Abbreviations

(v/v)	volume for volume
(w/v)	weight for volume
°C	degrees Centigrade
µg	microgram
µl	microlitre
µM	micromolar
aa	amino acid
AFC	antibody-forming cell
AMP	2-amino-2-methyl-1-propanol
APC	antigen-presenting cell (or allophycocyanin)
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BCR	B cell antigen receptor
bio	biotinylated
BSA	bovine serum albumin
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CFSE	5-(and6-)-carboxyfluorescein diacetate succinimidyl ester
DAB	3,3'-diaminobenzidine tetrahydrochloride
DC	dendritic cell
ddH_2O	distilled, deionised water (>18 MΩ resistance)
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylene diamine tetraacetate
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescence-activated cell sorter (or general flow cytometry)
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FO	follicular B cell subset
g	gram
g	gravity
H-2	MHC complex of mice
HEL	hen egg lysozyme
HEPES	*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethane sulfonic acid]

HRP	horse radish peroxidase
i.p.	intraperitoneal
i.v.	intravenous
Ig	immunoglobulin
IgM/G	chimeric BCR with IgM C_H and the IgG_1 membrane tail
IgM^a	IgM of the *a* allotype
IgMδE	chimeric IgM BCR with 27 aa extracellular spacer of IgD
L	Litre
LPS	lipopolysaccharide
MeOH	methanol
MHC	Major Histocompatibility Complex
ml	millilitre
mM	millimolar
MZ	marginal zone B cell subset
nM	nanomolar
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
pH	$-\log_{10}[H_30^+]$
PMSF	phenylmethylsulfonyl fluoride
RPMI	Roswell Park Memorial Institute
SA	streptavidin
TAC	Tris.ammonium chloride
TAE	Tris/acetate/EDTA buffer
TBS	Tris-buffered saline
TCR	T cell antigen receptor
TD	T (cell)-dependent
TEL	turkey egg lysozyme
TI	T (cell)-independent
Tween-20	polyoxyethylene sorbitan monolaurate

Chapter 1. Introduction

Section 1.1. Preamble

The immune system is a complex interconnected series of cell types and proteins that has evolved to protect the body from infection. The innate arm consists of physical and antimicrobial chemical barriers to infection as well as series of cells bearing receptors that recognise common conserved patterns amongst pathogens (Hoffmann, 1999). This is found in most forms of multicellular life and represents a complex but evolutionarily primitive form of immune system. By contrast, some form of adaptive immune system is shared by higher vertebrates and consists of cells that use recombined receptors to recognise a vast array of potential pathogen signatures. The hallmarks of adaptive immunity include a pathogen-specific response to clear infection and in many cases a state of immunological "memory" that provides long-term protective immunity against reinfection.

While an appreciation of the ability of the innate immune system to initiate immune responses and to regulate the other arms of the immune system has been relatively recent (Fearon, 1996), the adaptive arm of the immune system has been studied experimentally for over 100 years (Silverstein, 1989). The phenomenon of immune memory, especially with respect to antibody production by B lymphocytes, is well known and yet the underlying cellular mechanisms remain obscure. We know that a first encounter with a pathogen "primes" the immune system so that secondary, or memory immune responses have distinct qualitative and quantitative differences (Ahmed, 1996). Specific antibody is of higher affinity, is produced more rapidly and in much higher quantities compared to a primary response. In addition the antibody isotype profile changes, with switched isotypes such as IgG and IgA dominating. These complex, inter-connected changes make the phenomenon of "memory" very difficult to dissect, and it is still unclear what factors are responsible for the *maintenance* of the memory state versus the memory *response* itself.

For example, the role of isotype-switched B cell antigen receptor (BCR) expression on many memory B cells is unknown. At most other stages of B cell development it is well documented that signals through different molecular forms of the BCR influence B cell responses. During B cell production in the bone marrow, pre-BCR mediated signalling is crucial for developmental progression, while immature B cells respond to BCR crosslinking with clonal deletion, clonal anergy and a reduced life span. In the same way mature naïve B cells expressing IgM and IgD respond to protein antigen presented in the correct co-stimulatory context with clonal expansion, however in the absence of T cell help make an abortive response unless the antigen is highly multivalent or linked to a B cell mitogen. After a T-dependent immune response many isotype-switched memory B cells persist and are recruited into secondary antibody responses, however the contribution of

antigen-dependent signalling through isotype-switched receptors to the subsequent memory response has yet to be determined.

This introduction will review the role of BCR isotype in shaping B cell development, and outline the features of T-dependent immune responses. The unique qualities of the memory response and memory cells will be discussed, as well as current theories to explain the persistence of the memory state and the features of the memory response. Finally, evidence for isotype-specific signalling, both *in vitro* and *in vivo* will be reviewed and placed in context with the current study.

Section 1.2. B cell development and differentiation

B cell development from stem cells in the fetal liver and adult bone through to mature peripheral B cells can be thought of as a series of checkpoints where B cell antigen receptor (BCR) expression and signalling at each stage in B cell development plays an important role in shaping the eventual repertoire (reviewed in Rolink, 1993; Goodnow, 1995; Melchers, 1999; Pillai, 1999; Meffre, 2000; Monroe, 2000). B cell subsets in the bone marrow are shown in Figure 1-1, and peripheral B cell subsets in Figure 1-2.

1.2 (a) Stem cells and B lineage commitment

B lymphopoiesis in the mouse starts in the fetal yolk sac and moves to the fetal liver around day 14 of gestation. B lymphopoiesis is then established in the bone marrow in the first weeks after birth and continues for the life of the animal. In the bone marrow self-renewing hematopoietic stem cells give rise to common lymphoid progenitors that under normal conditions produce T, B and NK cells (Kondo, 1997). The stem cells and progenitor cells that give rise to lymphoid lineages are thought to express multiple different lineage determinants at low levels. In fact bone marrow-derived stem cells show remarkable plasticity and under the right conditions can contribute to a wide range of tissues including heart, brain, skeletal muscle and liver (Blau, 2001). Lineage "commitment" seems to involve the emergence of a more stable pattern of gene expression that reinforces lineage-specific gene expression and actively represses other possible cell fates (Nutt, 1999b; Akashi, 2000; Busslinger, 2000). In the case of B lymphopoiesis in the bone marrow, knockout studies have determined genes important for the development of B cells (Scott, 1994; Zhuang, 1994; Lin, 1995; Schilham, 1996; Wang, 1996). The transcription factor Pax-5 (also known as BSAP: B cell-specific activator protein) is the only factor shown so far to be required for B lineage commitment rather than

developmental progression. It is expressed from the earliest commited B cell stage, and while Pax-5$^{-/-}$ B cells appear to arrest at the early pro-B stage, they remain multipotent and able to form T cells, NK cells, osteoclasts, macrophages, dendritic cells and granulocytes *in vitro* (Nutt, 1999a) and in some cases *in vivo* (Rolink, 1999).

1.2 (b) B cell development in the adult bone marrow

The earliest stage of committed B cell precursors express the surface markers AA4.1, B220 and CD43 and are referred to as pre-pro-B cells (Hardy's Fraction A_0, A_1 and A_2) (Hardy, 1991; Li, 1993; Allman, 1999). These retain immunoglobulin loci in a germline configuration, express low levels of recombinase activator (RAG) genes and lack expression of components of the BCR, such as Ig$\alpha\beta$, suggesting initial B cell commitment is not driven by antigen receptor signals (Allman, 1999).

By contrast, pro-B cells (Hardy's fraction B and C) are the first B lineage stage to express a potentially functional precursor version of the BCR (the pro-BCR). This stage is distinguished by the expression of various additional surface markers such as CD24 (HSA)

and BP-1 (Hardy, 1991). During the pro-B cell stage sequential induction of heavy chain immunoglobulin $D_H \rightarrow J_H$ and $V_H \rightarrow D_HJ_H$ rearrangements occurs. Non-productive truncated forms of mIgμ (Dμ) are counter-selected at this stage leading to developmental arrest at the pro-B stage (Meffre, 2000). Pro-B cells express the Igα, Igβ, VpreB and λ5 components of the pre-BCR (Karasuyama, 1993; Meffre, 1996), and recent evidence suggests that a complex exists between calnexin and Igαβ on the surface of pro-B cells (Nagata, 1997). Interestingly, crosslinking of Igβ *in vivo* in RAG-deficient mice allowed the pro-B cells to display some features of pre-B cells as if the Igαβ complex on pro-B cells delivers a signal for developmental progression (Nagata, 1997). Further evidence that signalling *via* the calnexin/Igαβ complex may be important for pro-B cell progression comes from analysis of $Ig\beta^{-/-}$ (Gong, 1996) and μMT mice (Ehlich, 1993). In the absence of Igβ there are no pre-B cells, but in the pro-B subset $V_H \rightarrow D_HJ_H$ rearrangements are decreased compared to $D_H \rightarrow J_H$ joins. By contrast, in the absence of membrane-bound μ (and therefore a functional pre-BCR) there are a normal number of $V_H \rightarrow D_HJ_H$ rearrangements, suggesting that Igβ plays a role in signalling even in the absence of the pre-BCR. This observation fits with a model in which constant checks are imposed on developing B cells to ensure the correct expression of the components of a functional BCR.

After a period of clonal expansion, pre-B cells become small and resting (Hardy's fraction D). This is the stage of most light chain immunoglobulin gene $V_L \rightarrow J_L$ rearrangement, with a productively rearranged κ or λ light chain replacing surrogate light chain to form a complete BCR.

When a surface IgM isotype BCR is expressed, the B cell is considered "immature" (Hardy's fraction E). Immature B cells lose expression of IL-7R and have lower BP-1 expression (Hardy, 1991). The expression of a functional IgM BCR signals a cessation of Ig light chain gene rearrangement, the degradation of RAG proteins, and a loss of mRNA for RAG and pre-BCR components. Immature B cells exit from the bone marrow *via* the blood and mature further phenotypically in the spleen, eventually contributing to the mature, long-lived recirculating pool.

A fraction of bone marrow B cells are derived from the mature, recirculating pool that home back to this site. They are distinguished from the other bone marrow subsets by expression of mature B cell markers and are $CD23^+$, $B220^{hi}$, HSA^{lo} IgM^{lo} IgD^+ (Hardy's Fraction F) (Hardy, 1991).

1.2 (c) Selection shapes the mature B cell repertoire

Evidence for selection

Selection shapes the peripheral B cell repertoire during the transition from immature to mature B cells in the periphery, and also during the formation of specialised mature subsets in the splenic marginal zone and the peritoneal and pleural cavities. The fact that selection occurs at these points seems clear, however the relative importance of "negative" selection to self-antigens, foreign-antigen driven "clonal" selection or "positive" selection to undefined ligands for this process is still in doubt.

Only a small fraction of bone marrow-derived immature B cells are ever incorporated into the long-lived recirculating mature B cell pool (MacLennan, 1986). The exact site of cell loss has been difficult to determine and is probably a combination of apoptosis in the bone marrow and the spleen. Unlike the production of thymus-derived lymphocytes, which occurs in a defined organ and whose rate of emigration can be accurately estimated (Scollay, 1980), the actual rate of emigration of newly formed B cells from the bone marrow to the periphery is still uncertain. Estimates originally suggested a turnover rate of 2-5 x 10^7 IgM^+ B cells in the mouse bone marrow compartment daily (Opstelten, 1983; Osmond, 1986). While this may reflect the rate of cell production, it is likely to be a gross overestimate of the number of immature B cells that leave the bone marrow as many tolerance mechanisms operate at that site that lead to clonal deletion (Nemazee, 1989; Hartley, 1993). Also, because of the rapid clearance of apoptotic bodies in the bone marrow the rate of B cell apoptosis is difficult to determine. Recently the emigration rate of B cells from murine BM has been revised to 9x10^6 IgM^+ cells per day by taking into account the rate of apoptosis (Lu, 1997, 2000).

After entry into the spleen, there is evidence for two B cell populations, one with a very short turnover rate of a few days (Allman, 1993), and one with a half-life of several months (Gray, 1988; Forster, 1990; Allman, 1993; Hao, 2001). The prevailing view is that the short-lived subset represents immature B cells and that a very small fraction (1-10 %) of these enter the long-lived pool (Ron, 1985; Lortan, 1987; Gray, 1988; Forster, 1990; Chan, 1993), although the use of BrdU and other labels that mark proliferating cells rest on the assumption that immature B cells are the only cells derived from proliferating precursors. A subset of mature B cells are also likely to be proliferating by mechanisms such as clonal expansion due to encounter with both T-dependent and T-independent antigens (MacLennan, 1986; Chan, 1993), making the interpretation of labelling data difficult. The loss of the majority of recent bone marrow emigrant B cells that reach the spleen is probably due to their inability to enter B cell follicles and receive survival signals.

Certainly the "space" available for new B cells in the follicle niches is a critical, presumably neutral mediator of entry into the long-lived pool, as demonstrated by studies showing the rapid "re-filling" of those areas after depletion (Bazin, 1985b, 1985a). This "clonal competition" (MacLennan, 1986; Lortan, 1987) is also regulated by negative selection forces that affect the ability of self-reactive B cells to compete for follicle entry (Cyster, 1994).

The second piece of evidence for selection shaping peripheral B cell subsets comes from the restricted pattern of J558 V_H gene useage in mature splenic B cells compared to bone marrow derived immature B cells (Gu, 1991). In addition, the B-1 subset is enriched for B cells specific for numerous self and foreign antigens (Wortis, 2001) and recent analyses in rats (Dammers, 1999a) and several transgenic mouse models (Martin, 2000a) show that the splenic marginal zone compartment can be enriched for certain specificities.

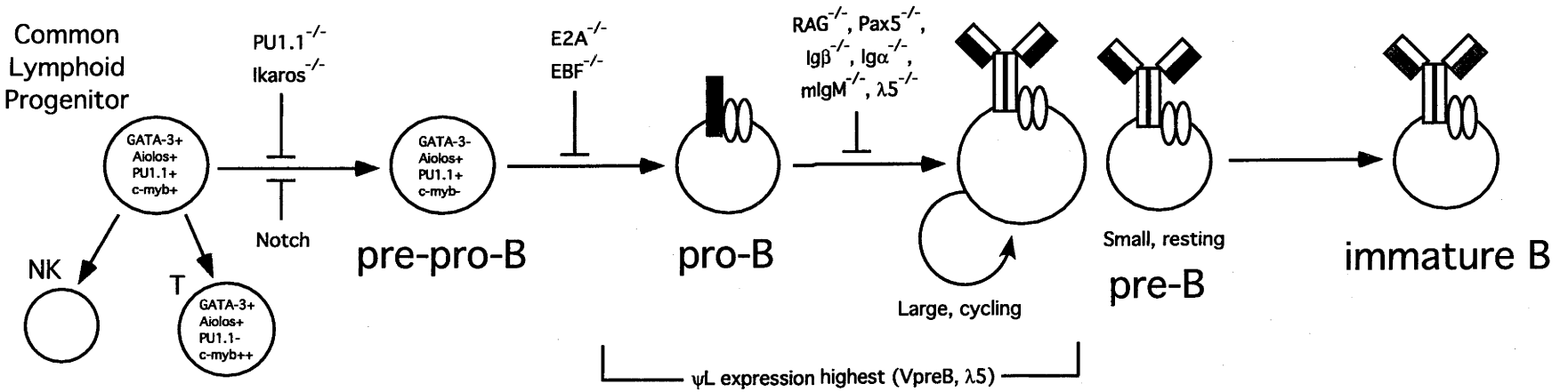
Negative selection

Negative selection is a major force in determining the nature of the mature B cell repertoire. The nature and timing of self-antigen exposure leads to a number of negative selection responses in B cells. This process has been most carefully studied in transgenic models that allow the regulated expression of autoantigens (Goodnow, 1992; Goodnow, 1995; Nemazee, 2000).

In the bone marrow, the binding of high avidity self antigen to self-reactive BCRs on immature bone marrow B cells can lead to autoreactive cell deletion (Nemazee, 1989). Self antigen binding to high avidity autoantigens causes developmental arrest at the immature B cell stage, which is separate from subsequent apoptotic loss (Hartley, 1993). It seems that strongly self-reactive immature B cells can alter their antigen receptors by secondary Ig gene rearrangements during developmental arrest, a process termed "receptor editing" (Radic, 1993; Tiegs, 1993). Subsequent expression of a non-autoreactive BCR then allows immature B cells to "escape" the developmental block, although this mechanism still produces an effective "clonal deletion" of the original self-reactive BCR. This process has been modelled in the 3-83 anti-H-$2^{k,b}$ transgenic system. On a $RAG^{+/+}$ background, "edited" mature B cells carrying endogenous Lc accumulate in the periphery, while on the $RAG^{-/-}$ background B cells fail to populate peripheral lymphoid organs (Spanopoulou, 1994).

Figure 1-1. B cell development in the bone marrow

This diagram outlines the major steps in B cell lymphopoiesis in the bone marrow, from hematopoietic stem cell precursors through pre-pro-B cells, pro-B cells, pre-B cells and immature B cells. The expression of relevant cell surface and intracellular markers is indicated by bars underneath each cell subset. Also, the nomenclature for each subset according to Hardy (Hardy, 1991; Li, 1993) is indicated A→E. Calnexin is shown in green in a putative receptor complex with Igαβ (yellow) on pro-B cells. Rearranged heavy chain is shown in outline, either in complex with ψL (red) on pre-B cells or in complex with rearranged light chain (blue) on immature B cells. Molecules involved in the transitions between the subsets are shown at the relevant points, where $x^{-/-}$ means the phenotype observed in the knockout of molecule *x*.



Nomenclature (Hardy's Fraction)	A	B	C	C'	D	E
B220+	←	—	—	—	—	→
CD43+	←	—	—	—	⊣	
HSA+		←	—	—	—	→
BP-1+		←	—	—	—	→
Hc locus	GL	D to J	V to DJ	V to DJ	VDJ	VDJ
Lc locus	GL	GL	GL	GL	V to J	VJ
Receptor type		pro-BCR	—	pre-BCR	—	BCR
RAG expression	Low	+	+	-	+	Low
Bcl-2 expression	Highest	—	—	—	—	Lowest

The importance of receptor editing versus cell death as a mechanism to ensure tolerance against high avidity, abundantly expressed self-antigens is unclear. While one report suggested that evidence of receptor editing is common amongst $IgM^+\lambda^+$ peripheral B cells in normal mice (Retter, 1998), most light chain gene rearrangements are to Jκ1 (Nishi, 1985), suggesting receptor editing is not a common occurrence, at least for κ^+ cells.

For the systemic membrane-bound anti-MHC system, as well as the anti-HEL system where HEL is expressed as a membrane-bound array, the major pathway of tolerance in the bone marrow is developmental arrest, followed by receptor editing or clonal deletion. By contrast, when a lower avidity soluble form of HEL is used, clonal deletion gives way to clonal anergy, in which many self-reactive B cells mature and fill the periphery but are functionally impaired (Goodnow, 1988; Goodnow, 1989; Goodnow, 1995; Goodnow, 1996). Anergic B cells in this system have been shown to have a reduced lifespan due to peripheral follicular exclusion (Cyster, 1994), are unable to up-regulate co-stimulatory molecules (Ho, 1994), and are actively killed *via* the Fas/FasL pathway by antigen-specific T cells (Rathmell, 1995; Rathmell, 1996). They express lower levels of BCR, flux calcium poorly in response to receptor crosslinking and only generate a subset of the transcriptional responses usually associated with full B cell activation (Glynne, 2000). Sufficiently low avidity systemic autoantigen can actually be ignored by the developing B cell, as in the case where a secreted form of $H\text{-}2K^b$ was expressed in mice carrying the 3-83 anti-$H\text{-}2^{k,b}$ Hc and Lc genes (Nemazee, 1991). Clonal ignorance has also been observed in anti-HEL double transgenic mice expressing sufficiently low concentrations of circulating HEL (Goodnow, 1989; Cyster, 1994).

Tolerance induction is not simply limited to the encounter of systemic autoantigen in the bone marrow. For example, restricted expression of membrane-bound $H\text{-}2^k$ in the liver led to the deletion of anti-$H\text{-}2^{k,b}$ 3-83 transgenic B cells after exit from the bone marrow, probably in the spleen during the transition to the mature cell compartment (Russell, 1991). To model negative selection in fully mature, peripheral B cells the Cre/loxP inducible gene-inversion system was used to switch BCR Hc expression on mature B cells from an innocuous specificity to recognise anti-$H\text{-}2^{k,b}$ (Lam, 1998). This sudden exposure of mature $3\text{-}83^+$ B cells to membrane-bound $H\text{-}2^b$ molecules led to very rapid elimination of those cells, indicating that sufficiently strong tolerising signals can negatively select even phenotypically mature B cells. In a similar way, the peritoneal cavity microenvironment of anti-erythrocyte transgenic mice allows auto-reactive B-1 cells to accumulate (Murakami, 1992), while B-2 cells are efficiently deleted. Injection of erythrocytes into the peritoneal cavity leads to B-1 cell deletion, again showing that mature B cells can be efficiently tolerised if auto-antigen exposure is sufficiently strong. Finally, the anti-TNP transgenic

system was used to model the effect of sudden antigen encounter on peripheral B cell subsets (Carsetti, 1993, 1995). It was found that the major B cell subset affected by antigen encounter were "transitional" cells in the bone marrow and spleen, leading the authors to speculate that immature "transitional" B cells in the bone marrow and spleen are sensitive to apoptosis and are primary targets for negative selection in the periphery.

At present it is unclear why immature B cells often respond to antigen binding with a tolerance response while mature B cells often respond to antigen binding with activation (Monroe, 2000). Extrinsic factors that lead to B cell tolerance must include a lack of appropriate T cell help and co-stimulatory signals during self-antigen binding. Also, the extrinsic microenvironmental differences between bone marrow and peripheral lymphoid tissue may play a role in determining whether immature B cells respond with developmental arrest (Melamed, 1998) or apoptosis (Carsetti, 1995). For example, the culture of immature B cells with unfractionated bone marrow protects immature cells from apoptosis due to BCR crosslinking (Sandel, 1999; Sandel, 2001). Intrinsic factors may also affect B cell responses to antigen. For example, antigen receptor crosslinking in an immature B cell line and in thymocytes does not induce antigen receptor movement into lipid microdomains (Ebert, 2000; Sproul, 2000), which is an important early event in full antigen receptor signalling in mature B and T cells (Ebert, 2000; Sproul, 2000). In a similar way, pro-apoptotic and anti-apoptotic members of the Bcl-2 family alter their expression levels during the immature to mature B cell transition, with Bcl-2 most highly expressed in pro-B and mature B cells, but poorly expressed in immature B cells (Li, 1993). Bcl-xL is expressed most highly in pre-B cells but is nearly absent from mature B cells (Choi, 1996). A1 expression is low in immature and transitional cells but is highly expressed in mature B cells (Tomayko, 1998). There is evidence that the relative levels of these anti-apoptotic members may make immature B cells more sensitive to cell death compared to mature B cells. Over-expression of Bcl-2 and Bcl-xL, for example, lead to the impaired deletion of autoreactive immature B cells in the bone marrow (Hartley, 1993; Fang, 1998).

Antigen-driven selection

Superimposed upon negative selection during B cell development is the process of antigen-driven selection by active immune responses that lead to antigen-specific B cell clonal expansion. This process, coupled with the culling of self-reactive clones by negative selection may be sufficient to explain the makeup of the mature B cell repertoire. In particular, the role of antigen-driven selection in promoting B cell entry into the marginal zone and B-1 peripheral subsets will be dealt with later.

Some researchers invoke a third process, termed "positive" or "ligand" selection, to explain observations such as V_H gene useage and clonal enrichment in certain B cell subsets (Gu, 1991; Martin, 1999; Pillai, 1999). In this model they envisage that B cells are selected and maintained on the basis of their specificity in the pre-immune repertoire by contact with some self or foreign antigen. It is certainly clear that peripheral B cell survival requires functional BCR expression, as conditional ablation of BCR expression leads to rapid mature B cell death (Lam, 1997). Also, survival signals can be transmitted through the BCR, for example when B cell survival in $CD45^{-/-}$ mice was rescued by binding self antigen (Cyster, 1996). However, in contrast to $CD4^+$ (Takeda, 1996; Kirberg, 1997) and $CD8^+$ (Tanchot, 1997) T cells, which require MHC contact for survival, there is no way at present to distinguish whether a BCR must contact some specific ligand or whether "tonic" BCR signalling is sufficient for survival. For B cells, which lack obvious common ligands and which could potentially interact with a vast array of different molecules with varying avidity, only one case has been reported in which the development of a peripheral B cell clone is dependent on the presence of a specific ligand (Hayakawa, 1999). In that study, the formation of anti-Thy-1 B-1a B cells was shown to be dependent on the presence of the Thy-1 antigen. While that study showed that expression of CD5 on a given B cell clone required the presence of the cognate antigen it failed to determine whether that interaction was a true positive selection signal or simply an antigen-driven phenotypic change.

In summary, it is unclear whether true positively selecting ligands act to shape the B cell repertoire. It seems the term "positive" selection is currently used loosely in B cell biology to include not only ligands that are absolutely required for the simple maintenance of mature lymphocytes (as in T cell biology), but also for the antigen-driven process of clonal expansion. Currently a combination of the processes of negative selection and antigen (self or foreign)-driven clonal expansion seems a simpler explanation for the patterns of selection observed in the B cell repertoire. It may be that genuine, pre-immune, positively selecting ligands, such as those operating on peripheral T cells may not operate in B cell biology.

1.2 (d) B cell subsets in the periphery

The formation of mature B cell subsets

After exit from the bone marrow, immature B cells move rapidly *via* the blood to the spleen (Osmond, 1986), where most are excluded from follicular entry (Lortan, 1987). Rather than automatically entering the mature peripheral pool, immature B cells undergo further maturation steps (called "transitional" stages) (Figure 1-2).

During these transitional stages IgD expression gradually increases, from $IgD^{lo/-}$ through to IgD^{hi}. In some studies this transition is divided into HSA^{hi} $B220^{lo}$ IgM^{+} IgD^{-} immature and HSA^{lo} $B220^{hi}$ IgM^{+} IgD^{+} mature subsets (Brink, 1992; Allman, 1993). Other studies use more comprehensive markers to define transitional type 1 (IgD^{lo}, IgM^{hi}, $CD21^{lo}$, $CD23^{-}$, $CD62L^{-}$), transitional type 2 (IgD^{lo}, IgM^{hi}, $CD21^{hi}$, $CD23^{+}$, $CD62L^{-}$) and mature B cell subsets (*follicular*: IgD^{hi}, IgM^{lo}, $CD21^{int}$, $CD23^{+}$, $CD62L^{+}$ and *marginal zone:* $IgD^{lo/-}$, IgM^{hi}, $CD21^{hi}$, $CD23^{-}$) (Carsetti, 1995; Loder, 1999). T1 and T2 subsets are absent from lymph nodes and the T2 phenotype is seen only in the spleen, consistent with the immature to mature B cell transition occurring almost exclusively in this location (Loder, 1999). T1 cells appear first during ontogeny, followed by T2, mature follicular and finally marginal zone cells. On the basis of cell transfer experiments, T1 cells give rise to T2 cells that are then capable of forming mature B cells (Loder, 1999).

The origin of the marginal zone subset is less clear. Some marginal zone B cells are memory cells, derived presumably from mature follicular B cells during T cell-dependent responses (MacLennan, 1990). As well, many naïve marginal zone B cells are derived from mature, recirculating B cells. Marginal zone B cells appear after mature B cells during ontogeny (Loder, 1999), appear after follicular B cells during reconstitution of hind limb shielded irradiated rats (Lane, 1986) and are rapidly repopulated by transfer of mature recirculating thoracic duct lymphocytes into marginal zone depleted rats (Kumararatne, 1981b). Despite these findings, both $CD45^{-/-}$ and Btk^{xid} mice show severe defects in mature follicular phenotype B cells (IgM^{lo} $CD21^{med}$ $CD23^{+}$), and yet have phenotypically normal marginal zone subsets (IgM^{hi} $CD21^{hi}$ $CD23^{-}$) (Loder, 1999). This raises the possibility that at some low level T1 or T2 cells can directly form marginal zone cells.

Mature, follicular B cells

Mature follicular (IgD^{+} IgM^{lo} $CD21^{med}$ $CD23^{+}$ $CD62L^{+}$) B cells constantly recirculate through secondary lymphoid tissues, blood and afferent lymphatic vessels and rely on functional BCR expression for their survival (Lam, 1997). They continue this process until triggered by antigen, a process that will be described further in the next section. As a population they have a half-life of the order of several months, based on BrdU labelling (Forster, 1990), disruption of IL-7 signalling (Grabstein, 1993; Sudo, 1993), and more recently gene-targeting experiments (Hao, 2001).

Marginal zone B cells

The marginal zone (MZ) of the spleen forms a boundary between the red pulp and the white pulp, consisting of the arteriole-draining marginal sinus with marginal zone

metallophilic macrophages on the follicular face and a ring of marginal zone B cells, dendritic cells and marginal zone macrophages as an outer boundary (Cyster, 2000; Vos, 2000). Blood constantly percolates through the MZ *via* the marginal sinus and outward into the red pulp and this close proximity to blood-borne antigens is thought to allow rapid MZ B cell activation during T-independent type 2 and memory B cell responses.

In normal mice, marginal zone B cells consist approximately 5-10 % of splenic B cells and are phenotypically distinct from other peripheral B cell subsets. They have been characterised as $CD21^{hi}$, $CD23^{-}$, IgM^{hi}, $IgD^{lo/-}$ (Gray, 1984b; Loder, 1999; Martin, 2000b) and $CD1d^{+}$ (Amano, 1998; Roark, 1998). In the mouse, marginal zone B cells may express higher levels of CD38, CD40 (Oliver, 1997a) and the co-stimulatory molecules B7.1 and B7.2 (Oliver, 1999) compared to follicular B cells. In addition, marginal zone B cells are non-dividing (Liu, 1988), enlarged (Oliver, 1997a) and non-recirculating (Kumararatne, 1981a; Kumararatne, 1981b).

The marginal zone B cell population of the spleen has been associated with two functions. Firstly, marginal zone B cells are involved in responses against T-independent type 2 antigens such as bacterial polysaccharides from encapsulated bacteria. Blood-borne versions of these antigens localise very rapidly to the area of the marginal sinus, trapped by resident macrophages (Humphrey, 1981; Gray, 1984a; Matsuno, 1989; Peset Llopis, 1996; Martin, 2000a). The key role of the marginal zone in allowing immune responses against these antigens is underlined by the fact that splenectomised patients and children under the age of 2 years, are unable to mount effective immune responses to encapsulated bacteria (Mond, 1995).

Because marginal zone B cells are able to form in the absence of T cells (Dammers, 1999a) and exposure to exogenous antigens (Kumararatne, 1981a), at least part of this subset can be considered naïve. However, B cells bearing evidence of somatic hypermutation also accumulate in the marginal zone compartment. In rats immunised with a T-dependent form of the hapten dinitrophenyl (DNP), hapten-binding memory B cells primarily localised to the marginal zone (Liu, 1991) and were rapidly mobilised from that site upon in an antigen-specific manner. In humans also, many marginal zone B cells show signs of a germinal centre origin by the presence of somatic mutation (Dunn-Walters, 1995; Tierens, 1999) and by the expression of cell surface markers such as CD27 which are associated with memory cells (Tangye, 1998). By contrast, when B cells from the spleen of unimmunised rats were analysed, only 20 % of cells were found to carry somatic mutations (Dammers, 1999b; Dammers, 2000). The difference in the extent of memory cells in the MZ of human and rodent spleen is difficult to interpret, as they are poorly matched comparisons.

The nature of the signals that lead to selection and maintenance of B cells in the follicular and marginal zone compartments is poorly understood. Memory B cells localise to the marginal zone, and the phenotypic changes that allow this have not been elucidated. As well, it has recently been shown that the pre-immune marginal zone repertoire is not random and may be subject to selective pressures. In one study by Dammers and colleagues, it was estimated that only 0.03 % of mature recirculating follicular B cells were used to replenish the marginal zone B cell pool in the rat per day (Dammers, 1999b). Evidence for the selection of B cell clones in this process came from an analysis of PC7183 family V_H genes in unimmunised rats (Dammers, 2000). Despite 80 % of B cells carrying germline-encoded sequences, it was found that the PC-1 and PC-4 family members were enriched in the follicular B cell compartment, and gene sequences from marginal zone B cells showed a shorter CDR3 region length compared to the other subsets tested. This correlation of short CDR3 length with marginal zone occupancy fits with the proposal of Martin and Kearney that the marginal zone is enriched by some form of "positive selection" for B cell clones with "polyreactive" antigen receptors (Martin, 2000a). They have shown using transgenic mice that extreme enrichment of certain clones into the marginal zone compartment can be obtained and requires intact antigen receptor signalling. The 81x heavy chain transgenic mouse carries a V_H81x gene derived from a multireactive mouse fetal liver B cell clone (Martin, 1997). The transgenic heavy chain preferentially associates with the Vκ1C/Jκ5 light chain in surviving peripheral B cells expressing the 81x Hc (Chen, 1997) and the resulting 35-1 idiotype$^+$ B cells constitute 94 % of the marginal zone subset and just 5 % of the follicular subset (Martin, 2000b). This enrichment process has also been shown in the M167 anti-phosphorylcholine Hc-only transgenic where M167 idiotype$^+$ B cells make up 83 % of the marginal zone subset and only 14 % of the follicular subset (Martin, 2000b). By contrast, the MD2 anti-HEL heavy-chain only line (Martin, 2000b) produces HEL-binding clones with a predominantly follicular phenotype.

Despite the striking accumulation of these clones in the marginal zone, the relative importance of negative and positive and their relevance for physiological selection in normal, diverse B cell repertoires is difficult to assess. There is evidence that V_H81x/endogenous Lc combinations other than Vκ1C/Jκ5 are the target of negative selection in the 81x transgenic model, including strong competitive exclusion in a diverse repertoire and a lack of transgene-encoded serum antibody or plasma cells (Martin, 1997). This negative selection may be offset in the case of "polyreactive" 81x/Vκ1C,Jκ5 clones by contact with some antigen such as bacterial products from commensal organisms, leading to the observed clonal enrichment through each stage of B cell development (Martin, 2000b). Certainly a lack of clonal enrichment in the absence of functional Btk suggests

some sort of positive signal *via* the BCR is required for the preferential accumulation of the 35-1 Id^+ cells in the marginal zone.

Despite the profound levels of selection shown in this transgenic model, clonal competition is also important for entry into the marginal zone pool. In bone marrow chimeras of 1:1 81x-Tg and non-Tg mixtures, only a tiny fraction of marginal zone cells (3 %) were derived from transgenic donor cells compared to nearly 100 % in normal 81x transgenic mice (Martin, 1997). Therefore it is possible that the extreme enrichment of clones into the marginal zone in the 81x Tg model is a transgenic artefact. The strong selection of 35-1 Id^+ cells in 81x transgenic mice may be due to negative selection of many 81x Hc/endogenous Lc combinations, allowing the 81x/ Vκ1C,Jκ5 bearing clones to enter and enrich the MZ subset by default. Under more normal conditions in the polyclonal, diverse B cell repertoire of the competition bone marrow chimeras, the selective forces that allow 35-1 Id^+ cell enrichment are swamped. This important competition effect is also seen in the anti-HEL system, where it has been noticed that in the presence of "competing" B cells of other specificities, the MD2 Hc-only anti-HEL line produces few HEL-binding marginal zone cells. By contrast, in the absence of B cells bearing enodogenous specificities, the MD4 Hc+Lc anti-HEL line produces a large number of HEL-binding marginal zone cells (Mason, 1992; Martin, 2000b).

Several signalling pathways have been implicated in the formation, selection and maintenance of the marginal zone B cell subset. The co-receptor CD19 seems to be essential for the production of marginal zone B cells (Martin, 2000b; Cariappa, 2001). By contrast, while expression of CD21 is important for the localisation of TI-2 antigens to the marginal zone (Guinamard, 2000), it is not required for the production of marginal zone B cells (Cariappa, 2000). The tyrosine kinase Btk (Bruton's agammaglobulinemia tyrosine kinase) has been implicated in the enrichment process by which certain specificities in the 81x and M167 transgenic lines accumulate in the marginal zone. Even though a marginal zone B cells can develop in Btk^{xid} animals, the enrichment process in 81x and M167 transgenic mice does not occur on a Btk^{xid} background (Martin, 2000b), suggesting that enrichment requires a certain level of BCR signalling. Cariappa and colleagues have presented a contrasting model in which "weaker" BCR-derived signals lead to more marginal zone B cells while "stronger" BCR-derived signals lead to an increased number of follicular B cells (Cariappa, 2001). In their view, the fact that $Btk^{-/-}$ mice show an greater ratio of marginal zone relative to follicular B cells suggests that marginal zone B cells develop to a greater extent when BCR signalling has been decreased. They also argue that marginal zone B cells are absent from $Aiolos^{-/-}$ mice, due to the fact that a negative regulator of BCR signalling has been lost so follicular B cell formation is favoured. The

importance of signalling through BCR-associated mechanisms for recruitment into the marginal zone is also suggested by the finding that in anergic MD4 x ML5 mice, anti-HEL transgenic B cells constantly exposed to a self antigen are depleted from the marginal zone. By contrast, in the naïve MD4 line anti-HEL transgenic B cells form a normal marginal zone subset (Mason, 1992). In this case decreased BCR signalling through the anergic BCR leads to fewer anti-HEL marginal zone B cells.

Other signalling pathways that are important for the formation and maintenance of marginal zone B cells involve the tyrosine kinase Pyk2, the GTP exchange factor (GEF) Lsc, trophic signals *via* BAFF receptors (BCMA, TACI and BAFF-R) and signals *via* NFκB.

The tyrosine kinase RAFTK/Pyk2 is related to Focal Adhesion Kinase and has been implicated in signalling by chemokine receptors, integrins, antigen receptors and ion channels (Avraham, 2000). In the absence of Pyk2, peripheral B cells show an intrinsic defect in marginal zone formation and fail to undergo chemotaxis to chemokines, suggesting Pyk2 may be part of the pathway that directs marginal zone B cells to or retains marginal zone B cells within their correct anatomical location. Connected to this is the finding that mice deficient for the GEF Lsc show reduced marginal zone B cells due to a lymphocyte-intrinsic defect (Girkontaite, 2001). This may be because normal marginal zone localisation requires the arrest of cell movement in response to blood-borne products, and $Lsc^{-/-}$ mice show a defect in the serum-dependent inhibition of chemotaxis.

Marginal zone B cells are in close proximity with marginal zone macrophages and metalophilic macrophages, and the finding that the B cell survival factor BAFF (also known as BLyS, TALL, THANK, zTNF4) is expressed on monocytes, macrophages and dendritic cells (Moore, 1999; Schneider, 1999; Shu, 1999) raises the possibility that interactions with other cells is required for marginal zone B cell function. Interestingly, transgenic mice that over-express BAFF show B cell hyperplasia, particulary in the transitional type 2 and marginal zone compartments (Mackay, 1999; Batten, 2000). The signalling pathways responsible for the action of BAFF are not fully known, although reports suggest that BAFF is able to activate NF-κB and JNK (Do, 2000; Xia, 2000; Yan, 2000). An absence of marginal zone B cells has been reported in $NF\kappa Bp50^{-/-}$ mice (Cariappa, 2000) and it may be that BAFF overexpression mediates its effects on marginal zone B cells *via* NFκB.

B-1 B cells

The study of B-1 phenotype B cells has been dominated by two competing theories. One, the "lineage" hypothesis, maintains that B-1 cells represent a distinct lineage, abundant in fetal liver hematopoietic precursors but absent from adult bone marrow (Herzenberg, 2000). The alternative "instructive" theory maintains that the B-1 pool is derived from the same lineage as B-2 phenotype cells due to differences in antigen contact, selection and compensatory phenotypic changes (Wortis, 2001). Recent studies outlined below show the remarkable plasticity of phenotype choice among peripheral B cell clones in different situations, lending weight to the "instructive" hypothesis.

B-1 B cells have a distinctive phenotype, they are self-renewing and are enriched in the pleural and peritoneal cavities of adult mice. They are $CD5^+$ (B-1a only, B-1b cells are $CD5^-$), IgM^{hi}, IgD^{lo}, $CD23^{lo/-}$, $CD43^+$, $B220^{lo}$ and $CD11b^{hi}$ (in the peritoneal cavity only) (Hayakawa, 1983). In contrast to B-2 follicular B cells, the B-1 pool is enriched for B cell specificites directed against self antigens and bacterial antigens (Fagarasan, 2000b; Martin, 2000a). These are directed against foreign antigens such as bacterial phosphorylcholine, or against self antigens such as phosphatidylcholine, immunoglobulin, DNA, and membrane proteins on erythrocytes and thymocytes (Fagarasan, 2000a; Herzenberg, 2000; Martin, 2000a). B-1 cells are thought to be important for the production of so-called "natural" IgM antibodies present in germ-free animals and for IgA production against commensal bacteria across intestinal mucosal surfaces (Macpherson, 2000), as well as T-independent responses to blood-borne antigens (Martin, 2001).

The B-1 subset represents a more heavily selected subset than normal mature follicular B cells and selection of specific clones into the B-1 subset is exquisitely sensitive to alterations in BCR signal transduction. For example, mutations in negative regulators of BCR signalling such as SHP-1 (Cyster, 1995) and CD72 (Pan, 1999) tend to increase the B-1 pool, while mutations in positive regulators of BCR signalling such as Btk (Khan, 1995), CD19 (Engel, 1995; Inaoki, 1997), Cr2 (Ahearn, 1996), BLNK (Xu, 2000) and PLC-γ2 (Hashimoto, 2000) decrease the B-1 pool. Among individual clones this effect is clearly seen. For example, the anti-HEL MD4 transgenic contains essentially no B-1 antigen-specific cells. When BCR signalling is altered by the removal of the negative regulator SHP-1, many antigen-specific B-1 cells appear in the peritoneal cavity (Cyster, 1995). The modulation of BCR signalling in converting B-1 and B-2 B cells can work both ways. For example, transgenic mice expressing a rearranged V_H12 heavy chain generate predominantly B-1 B cells. Decreasing the strength of BCR signalling by crossing onto a Btk^{xid} background leads to most B cells adopting a B-2 phenotype (Clarke, 1998). The finding that certain transgenic clones can be manipulated to generate both B-1 and B-2

phenotypes and the finding that *in vitro* stimulation of B-2 cells can lead to upregulation of CD5 (Cong, 1991), suggests that antigen-driven selection is a major factor directing B cell entry into the B1 subset. The importance of antigen contact for recruitment of anti-thymocyte B-1 cells into this pool was elegantly demonstrated by Hayakawa and colleagues using heavy chain transgenic animals crossed to Thy-1 deficient mice (Hayakawa, 1999). In the absence of the selecting antigen, B-1a Thy-1 reactive cells were absent.

1.2 (e) Memory B cells

Memory B cells represent a poorly defined subset that are traditionally described as antigen-experienced B cells responsible for heightened "memory" or "recall" antibody responses to thymus-dependent (TD) antigens. The generation, maintenance and phenotypic and functional characteristics of memory B cells will be dealt elsewhere (see section 1.4 (b)). Recently the B-1 and marginal zone B cell subsets have been termed "natural memory" cells, on account of these subsets being enriched for both self-reactive and foreign-reactive B cell clones (Martin, 2000a). This is an extension of an earlier idea the evolutionary pressure has maintained certain B cell specificities because of their ability to react to common pathogens (Decker, 1992; Klinman, 1998). As discussed previously in section 1.2 (d), there is good evidence that antigen exposure is responsible for the enrichment of self and foreign reactive cells in the B-1 subset. Marginal zone B cells also have an activated phenotype, make a rapid response to blood-borne antigens and may be selected into that subset by prior antigen contact. Another traditional "memory" feature, that of long lifespan, may also be shared by B-1 and marginal zone subsets. When the production of new B cells in the bone marrow is disrupted in adult mice by the conditional targeting of the RAG2 gene, both B-1 and MZ subsets persist at stable levels (Hao, 2001). This may be due to continual cell homeostatic cell division rather than intrinsically long lifespans, but the effect is the same – a pool of antigen-reactive cells poised to respond to infection.

1.2 (f) Plasma cells

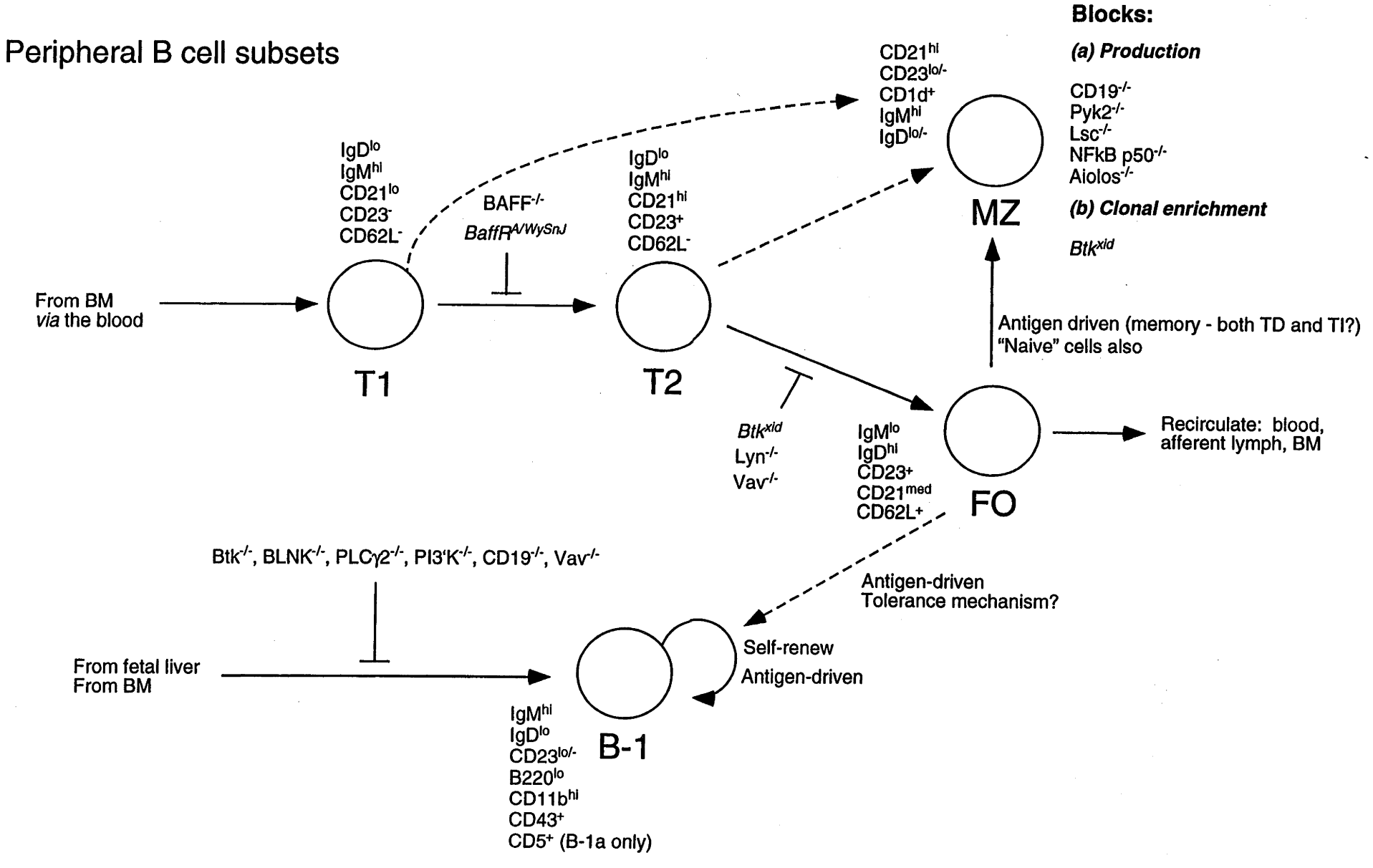
Plasma cells (PC) represent the terminally-differentiated effector stage of the B cell lineage. Phenotypically they are dedicated to the secretion of antibody and are $B220^{lo}$ (Smith, 1996), syndecan-1^{+} (Sanderson, 1989), $CD38^{hi}$ (human) (Arpin, 1997), $CD38^{-}$ (mouse) (Oliver, 1997b; Ridderstad, 1998), Class II MHC^{-}, J-$chain^{+}$, surface-$Ig^{lo/-}$ (Smith, 1997) and immunoglobulin-secreting. PCs are formed by two major routes during immune responses. TI and TD antigens both induce the early formation of PCs in extrafollicular foci and the red pulp of the spleen or the lymph node medullary cords. The majority of

these PCs are short lived (Ho, 1986), with most undergoing apoptosis *in situ* (Smith, 1996). In addition, TD antigens are also capable of driving the formation of germinal centres, which also lead to PC production and the subsequent production of high affinity antibody. Most PC derived from germinal centres home to the bone marrow, have a longer lifespan (Ho, 1986; Manz, 1997; Slifka, 1998b), and are thought to be critical for the maintenance of long-term protective immunity (Benner, 1981; Slifka, 1998a). The localisation of PC in extrafollicular sites in secondary lymphoid tissue as well as the bone marrow has recently been attributed to changes in chemokine responsiveness. PCs lose chemotactic responses to CXCL13, CCL19 and CCL21, while becoming more responsive to CXCL12, the CXCR4 ligand expressed in extrafollicular sites in the spleen and lymph node, as well as in the bone marrow (Hargreaves, 2001).

B cell terminal differentiation involves reciprocal regulation of the transcription factors Pax-5 and Blimp-1. Pax-5 is downregulated during terminal differentiation (Morrison, 1998) and over-expression of Pax-5 *in vitro* in B cell lines represses expression of syndecan-1 and high levels of Ig secretion (Usui, 1997). Blimp-1, by contrast, drives plasma cell differentiation, both *in vitro* (Turner, 1994) and *in vivo* (Angelin-Duclos, 2000), in part by repressing expression of c-myc (Lin, 1997). Plasma cell differentiation can be postponed in some circumstances, for example during the germinal centre reaction, where activated B cells undergo rounds of selection and hypermutation rather than terminal differentiation. It seems that Bcl-6, a transcription factor expressed highly in germinal centre cells (Shaffer, 2000; Shaffer, 2001) actively represses Blimp-1 expression, halting terminal differentiation (Reljic, 2000). This may be the mechanism behind observations that combinations of a variety of signals likely found in the germinal centre (BCR, CD40 ligation, some cytokines) can inhibit terminal differentiation (Randall, 1998; Knodel, 2001). The extrapolation of these *in vitro* studies to the selection environment of the germinal centre is difficult, however, especially given the tightly regulated, transient expression of these selecting factors *in vivo* (Yellin, 1994; Casamayor-Palleja, 1995).

Figure 1-2. B cell subsets in the periphery

This diagram shows the phenotype and putative relationships between the various B cell subsets of the periphery, from transitional type 1 recent emigrants to the mature follicular and marginal zone subsets. Signalling mutants in which the formation of these subsets is disrupted are indicated, as is the cell surface phenotype of each subset. Solid lines represent development pathways that are well understood, dashed lines represent less well characterised transitions.


Peripheral B cell subsets
Blocks:
(a) Production
CD19-/-
Pyk2-/-
Lsc-/-
NFkB p50-/-
Aiolos-/-
(b) Clonal enrichment
Btkxid
CD21hi
CD23lo/-
CD1d+
IgMhi
IgDlo/-
MZ
IgDlo
IgMhi
CD21lo
CD23-
CD62L-
T1
BAFF-/-
BaffRA/WySnJ
IgDlo
IgMhi
CD21hi
CD23+
CD62L-
T2
From BM
via the blood
Antigen driven (memory - both TD and TI?)
"Naive" cells also
Btkxid
Lyn-/-
Vav-/-
IgMlo
IgDhi
CD23+
CD21med
CD62L+
FO
Recirculate: blood,
afferent lymph, BM
Btk-/-, BLNK-/-, PLCγ2-/-, PI3'K-/-, CD19-/-, Vav-/-
Antigen-driven
Tolerance mechanism?
From fetal liver
From BM
Self-renew
Antigen-driven
IgMhi
IgDlo
CD23lo/-
B220lo
CD11bhi
CD43+
CD5+ (B-1a only)
B-1

Section 1.3. B cells in primary immune responses

Naïve mature B cells are capable of two main pathways of differentiation. During an immune response they are induced by a variety of signals to become either memory B cells, or to terminally differentiate into antibody-secreting plasma cells.

1.3 (a) T-independent immune responses

B cell responses to some antigens do not require thymus-derived MHC Class II-restricted αβ T cells. The antigens that cause TI responses comprise polyclonal B cell activators such as LPS, and antigens such as bacterial polysaccharides composed of repeated antigenic epitopes thought to induce strong BCR cross-linking (Mond, 1995). These antigens do not normally induce classical B cell memory, but may induce isotype switching, predominantly producing IgM and IgG_3. This thesis deals with a T-dependent system, so the rest of this introduction will describe properties of B cells associated with TD antigens.

1.3 (b) T-dependent immune responses

In contrast with TI antigens, T-dependent (TD) antigens are characterised by their ability to associate with MHC molecules on the surface of activated antigen-presenting cells to allow antigen-specific T cells to be recruited into an immune response. Through recruiting antigen-specific B cells, the TD response leads to immunological memory, protective immunity and high affinity antibody production through antigen-driven selection in germinal centres.

Activation of antigen-specific B and T lymphocytes

TD responses are initiated by antigen trafficking from sites of infection to secondary lymphoid tissue by dendritic cells (DCs) in peripheral tissues that take up antigen as they sample the local environment. Through "danger" signals associated with the infectious agents such as inflammatory chemokines and microbial products DCs are induced to mature functionally, upregulate co-stimulatory molecules and migrate to the T cell-rich areas of local lymphoid organs (Cyster, 1999). Migration of activated DCs to the T-cell rich paracortex of lymph nodes and the periarteriolar lymphoid sheath (PALS) of spleen facilitates antigen-mediated recruitment of $CD4^+$ helper T cells into the response.

Antigen-specific helper T cells are primed by DCs *via* a lengthy specific engagement of T cell receptor with peptide bound to MHC Class II molecules on the surface of the DC (Grakoui, 1999; Bromley, 2001). T cell priming leads to increased responsiveness to the chemokine CXCL13 and decreased responsiveness to the chemokines CCL21 and CCL19 (Ansel, 1999). This allows activated T cells to accumulate near the boundary of the B cell follicle and PALS at the site of initial contact with antigen-stimulated naïve B cells (van den Eertwegh, 1993; Toellner, 1996; Garside, 1998). This change in chemokine responsiveness also allows the eventual accumulation and expansion of helper T cells in the follicle required to sustain germinal centres (Kearney, 1994; Gulbranson-Judge, 1996; Ansel, 1999; Garcia de Vinuesa, 2000). Naïve antigen-specific B cells, by contrast, are activated by exposure to soluble antigen picked up from the blood or afferent lymph, and also change their distribution pattern to home to the border of the B cell follicle and PALS, probably in response to chemokine gradients (Cyster, 1999). Antigen-specific B cells are able to trap and process antigen to helper T cells in a highly specific way (Lanzavecchia, 1985), but in the naïve state are poor APCs, lacking co-stimulatory function and requiring primed T cell help (Parker, 1993).

As well as antigen-specific contacts, both T cell and B cell activation requires a variety of positive and negative "co-stimulatory" signals. These reinforce the collaborations made by DC, T and B cells and are essential for the normal spectrum of TD antibody responses by ensuring the full activation of antigen-specific cells. Positive "co-stimulatory" pairs important for the priming phase of a TD antibody response include CD28/B7.1, B7.2 (Freeman, 1993a; Freeman, 1993b; Shahinian, 1993; Borriello, 1997), CD40/CD40L (Foy, 1993; Kawabe, 1994; Renshaw, 1994; Xu, 1994; Foy, 1996), OX40/OX40L (Murata, 2000) and ICOS/B7h (B7RP-1) (Swallow, 1999; Yoshinaga, 1999; Dong, 2001; McAdam, 2001; Tafuri, 2001). Co-stimulatory signalling also provides for negative regulation of immune responses *via* related molecules such as CTLA-4 (Tivol, 1995; Waterhouse, 1995) and PD-1 (Nishimura, 1999). In addition, censoring mechanisms are active to stop the activation of overtly self-reactive cells. For example, anergic B cells are deficient in providing co-stimulatory signals to T helper cells (Ho, 1994), and so are unable to prevent apoptotic cell death *via* Fas ligation (Rathmell, 1995; Rathmell, 1996).

Extrafollicular antibody responses

The interaction of antigen-specific B cells with T cell help leads to two pathways of B cell differentiation. Extrafollicular foci of antigen-specific plasmablasts form relatively early during many responses near bridging channels (also known as "junction zones") where the splenic marginal zone intersects the PALS (Jacob, 1991; Liu, 1991; Jacob, 1992;

Sze, 2000). These foci differentiate into plasma cells over the next 2-6 days and produce unmutated, low affinity antibody (Smith, 1997), but most are lost from the spleen in a wave of apoptosis shortly afterward (Smith, 1996). A few plasma cells can persist in this site for several weeks (Ho, 1986; Sze, 2000). This early production of antibody is important for defence against infection, and for providing immune complexes to stimulate other arms of the immune system, allowing antigen to be trapped by follicular dendritic cell networks.

The signals that direct B cells to the extrafollicular differentiation pathway are poorly understood. In one study using polyclonal blocking antisera against OX40 it was proposed that T cell OX40 and B cell OX40L interaction was required for the development of extrafollicular foci but not for germinal centres (Stuber, 1996). Recently this study has been reinterpreted by Lane and colleagues, who believe that the blocking sera used in the previous work was engaging OX40 on activated T cells and driving them into a follicular pathway of differentiation (Walker, 1999). It is tempting to speculate that even if OX40/OX40L signalling is not directing B cells to the extrafollicular reaction, the temporally regulated movement of most activated T cells into B cell follicles (Kearney, 1994; Gulbranson-Judge, 1996; Ansel, 1999) sets a time limit for the recruitment of antigen specific B cells into the extrafollicular response. The other critical factor is probably the availability of free antigen that is complexed quickly by the first wave of antigen-specific antibody from the foci themselves (Liu, 1991).

Plasmablasts in extrafollicular foci do not associate with antigen-specific T cells (Jacob, 1991; Gulbranson-Judge, 1996) and the reaction does not require CD40 ligation for maintenance (Han, 1995a). Instead, myeloid-derived CD11c^{hi} "plasmablast-associated" dendritic cells (PDCs) have been co-localised with extrafollicular foci, and experimental manipulations that increase the number of these PDCs tend to increase the ability of the spleen to support plasma cells (Garcia de Vinuesa, 1999). This may have relevance to the extrinsic factor that places a ceiling on the capacity of the spleen to support plasma cells, at least in the response to the hapten NP (Sze, 2000).

Much of our knowledge about the extrafollicular focus reaction comes from the response of mice to the hapten NP. Other model systems, such as the primary anti-Ars response in A/J mice (Vora, 1998) or the anti-DNP primary response in rats (Liu, 1991) do not generate many foci. Also, many anti-protein primary responses do not generate significant antigen-specific IgM (Metzger, 1984; Nossal, 1989). The reasons behind this diversity probably include different antigen-specific precursor B cell frequencies in unimmunised mice (Press, 1974; Stashenko, 1980; Toellner, 1996), as well as varying grades of tolerance to protein and hapten antigens which may cross-react with self-antigens to some degree (Nossal, 1989; Nossal, 1990; Vora, 1998).

Germinal centre reactions

Germinal centres (GCs) are critical for generating high-affinity memory B cells and long-lived plasma cells during TD responses (MacLennan, 1994; Kelsoe, 1995). They begin to form ~ 4 – 10 days after the immune response is initiated (Liu, 1991) when as few as 3-5 (Liu, 1991; Jacob, 1992) B cell blasts move from the boundary of the B/T cell zones into B cell follicles and begin a process of exponential expansion, with a cell division rate of ~6-7 hours (Zhang, 1988; Liu, 1991). The signal required for the initial nucleation of the GC is not understood, as the centroblast pool can form in the absence of T cell help (Garcia de Vinuesa, 2000) and functional GCs can form in the absence of detectable immune complex deposition (Hannum, 2000). Anatomically, mature GCs consist of a "dark zone" of centroblasts that undergo somatic hypermutation to generate the raw material upon which antigen-driven selection takes place. Centroblasts that exit cell cycle and re-express somatically mutated antigen receptors are known as centrocytes. These move into the "light zone" of the germinal centre that is filled with antigen-specific T cells, follicular dendritic cells and tingible body macrophages, where the processes of selection and affinity maturation take place (MacLennan, 1997).

The somatically mutated centrocytes are thought to compete for access to antigen depots on FDCs and then receive "rescue" signals from antigen-specific T cells, which make up 5-10 % of the GC (Gray, 1997) and are located toward the outer face of the "light zone" (Casamayor-Palleja, 1995). There is evidence that GC-resident T cells have special qualities, such as the ability to rapidly mobilise CD40L expression upon TCR engagement (Casamayor-Palleja, 1995), as well as distinct cytokine and co-stimulatory profiles (Breitfeld, 2000; Schaerli, 2000; Kim, 2001). The CD40/CD40L pathway has been implicated in this process, as *in vivo* blockade using mAb led to the rapid dissolution of established GCs (Han, 1995a). After interaction with antigen specific T cells three choices are available for the B cell. Some probably re-enter the centroblast pool to undergo further rounds of somatic mutation to sustain the germinal centre (Casamayor-Palleja, 1996; Garcia de Vinuesa, 2000). Alternatively, memory B cells or long-lived plasma cells secreting high-affinity antibody can be formed. The nature of the selective signals that determine memory cell versus plasma cell differentiation are poorly understood, however *in vitro* reports suggest that the relative engagement of CD40 on human GC B cells determines whether the memory or plasma cell pathway is induced (Arpin, 1995).

Apoptosis is the default pathway for B cells that have low affinity or overtly self-reactive antigen receptors (Han, 1995b; Pulendran, 1995; Shokat, 1995). The signals leading to apoptosis of lower affinity and autoreactive B cells is obscure. An early report suggested that Fas-mediated apoptosis was not a factor, despite expression of Fas/FasL in

the GC, as *Fas*^{lpr} mice still produced memory B cells and high affinity AFCs (Smith, 1995). A recent study has re-opened the possibility that Fas does play a part in selection, as memory B cells in *Fas*^{lpr} mice accumulate many more somatic mutations (Takahashi, 2001). The role of Bcl-2 in the selection process is also interesting, as Bcl-2 overexpression increases the recruitment of lower-affinity memory B cells (Smith, 1994), but the selection of high-affinity bone marrow AFCs is not affected. This may point to different antigen-driven selection processes, one involving a "competitive" selection of memory cells that can be overcome by simply inhibiting apoptosis, and the other involving a "threshold" affinity selection of plasma cells (Smith, 2000).

Isotype switching: mechanism and signals

Isotype switching is the process by which antibody production is diversified to include a range of alternative effector functions as well as allowing expression of different molecular forms of the BCR. The switch of antibody isotype profile from IgM to IgG_1 especially is a hallmark of the TD response to protein and hapten antigens (Nossal, 1989; Toellner, 1996).

Switching involves the recombination of immunoglobulin heavy chain variable region gene segments upstream of alternative constant region genes, and is an irreversible differentiation step. In the mouse, the IgH locus on chromosome 12 is arranged with heavy chain constant region exons in the order 5' μ - δ - γ3 - γ1 - γ2b - γ2a - ε - α 3' (Coffman, 1993). Switch regions consisting of GC-rich tandem repeats located upstream of every constant region section (except δ) provide the sites for recombination, which most commonly involves the excision of a DNA loop containing the heavy chain constant region genes between the donor and acceptor points. In this way secondary switch recombination to downstream constant region genes is possible but upstream gene segments are lost.

The specificity of switch recombination lies in the signals that regulate the production of germline transcripts from the various downstream contant regions. Many of these are derived from cytokines and have been studied extensively *in vitro*, for example, in murine B cells IL-4 promotes IgG1 and IgE switching and IFN-γ promotes IgG2a and IgG3 switching (Snapper, 1987; Coffman, 1993; Stavnezer, 2000). *In vivo* evidence for the effect of these cytokines on switch recombination includes studes in knockout mice (Kuhn, 1991) and the cytokine-mediated deviation of normal isotypes in model immune responses (Finkelman, 1988; Buchanan, 1995). Engagement of CD40 on B cells by CD40L on activated T helper cells is also required for isotype switching to isotypes produced during TD responses. This is highlighted by the absence of switched isotypes in humans (Allen,

1993; Aruffo, 1993; DiSanto, 1993; Korthauer, 1993) and mice (Allen, 1993; Aruffo, 1993; DiSanto, 1993; Korthauer, 1993) with crippled CD40 signalling pathways.

There may be multiple sites of isotype switching during a normal TD immune response. An early report suggested that switching took place in the germinal centre, on the basis that the majority of germinal centre PNA^{hi} cells were IgM^+ in the primary response but IgG^+ in the secondary response to heterologous red blood cells (Kraal, 1982). In keeping with this, analysis of sorted subsets of human tonsillar B cells suggested that germline transcripts were found most abundantly in germinal centre centrocytes (Liu, 1996b) and it was hypothesised that switching at this point reduces the risk of potentially self-reactive B cells acquiring new effector functions. The chronic immune response of the human tonsil is difficult to compare with response in experimental animals and data from mice immunised with hapten-carrier conjugates is more consistent with the induction of isotype switching prior to entry into germinal centres. Jacob *et al.* showed that isotype switching in extrafollicular foci was relatively synchronous in NP-CGG primary responses and all germinal centres were populated by IgG^+ cells (Jacob, 1991). In a similar study, Toellner *et al.* showed that the induction of γ1 germline transcripts was a very rapid event that coincided with B/T collaboration in the outer PALS, at least for CGG-specific memory cells (Toellner, 1996). The earlier induction of switch recombination in this model is proposed to be a more effective way of producing IgG, as switch recombination occurs prior to major periods of clonal expansion at extrafollicular sites or germinal centres.

The remnants of an immune response – memory B cells and long-lived plasma cells.

One of the most striking features of the adaptive immune system is the ability to provide protective immunity for many years after a primary immune response. This is a combination of the presence of antigen-specific "memory" cells and the high titres of antigen-specific antibody provided by long-lived plasma cells in the bone marrow, both of which will be considered in more detail in the next section.

Section 1.4. Memory cells and memory responses

Humankind has been familiar with the concept of immune memory for over 2000 years and it has been frequently noted that those who survived a disease were often "exempt" ("immunatis") from its effect during a second epidemic (Silverstein, 1989). Early scientific observations of long-term immunity include the famous case of two

measles epidemics on the remote Faroe Islands. Ludwig Panum, a Danish physician observed that no-one affected by measles in the 1781 epidemic was afflicted with the disease during a second epidemic in 1846, 65 years later (Matzinger, 1994; Ahmed, 1996).

Despite the very real practical benefit of immune "memory" in the successful design of vaccines, a clear understanding of its cellular and molecular basis is still elusive. There is significant debate about the mechanisms of immune memory and the mechanisms for the persistence of the memory state.

1.4 (a) Early observations on the nature of immune memory

Memory responses to TD antigens are characterised by the rapid formation of high titres of high affinity isotype-switched antibody, and these features have been observed experimentally since the early 1900s (Glenny, 1931; Freund, 1953). In his writing on "Active Immunisation with Toxin", Glenny states:

> "The essential difference between primary and secondary stimulus response lies in the duration of the latent period and in the amount of antitoxin produced." (Glenny, 1931)

Experiments in a number of different animal models showed that secondary antibody responses were much more rapid than primary responses and led to much higher titres of antigen-specific antibodies (Burnet, 1949; Freund, 1953; Dixon, 1954; Burnet, 1959). In addition, the dominance of IgG over IgM in the secondary response was noted. Starch block electrophoresis (Tiselius, 1938) and analytical ultracentrifugation (Heidelberger, 1936) of serum proteins showed pre-immune sera contained a predominant antibody species of ~180,000 Da (sedimentation coefficient 7S) and a minor antibody form of ~$1x10^6$ Da (sedimentation coefficient 18S). The relative levels of these antibody species were found to vary during the course of an immune response and a switch from 18S (IgM) to 7S (IgG) antibody production was described using a number of protein (Bauer, 1961) and particulate antigens (Stelos, 1957; Uhr, 1962).

Early models viewed the changes during the secondary response as being due to the physical release of pre-synthesised antibody, rather than fresh antibody production (Pauling, 1940). An important conceptual change came from the views of Burnet and Fenner (Burnet, 1949) in which the logarithmic rise in antibody during a secondary response was interpreted as evidence for the multiplication of antibody-producing units in the body. This was further developed in Burnet's "Clonal Selection Theory" of antibody formation (Burnet, 1959) in which clones of cells carrying antigen-reactive receptors were selected to participate in an immune response. Burnet provided a simple explanation for memory responses with the statement:

"The primary stimulus finds only a few examples of the appropriate clones; by the time of the secondary stimulus many more individuals of the selected clones are accessible." (Burnet, 1959)

This is an important theme in immune memory that is still debated, as it is still not clear to what extent memory responses can be explained by the increased clonal precursor frequency of reactive cells resulting from prior contact with antigen (Celada, 1971; Freer, 1995; Ahmed, 1996). Some reports concluded that the rate of clonal expansion and antibody production on a per cell basis was equal during the primary and secondary immune responses (Uhr, 1961). Burnet hinted, however, that he did not accept this simple explanation, and described the primary response as a "tooling-up" event that alters the immune system in a more fundamental way to account for the special characteristics of the secondary response (Burnet, 1959).

After the discovery that the small lymphocyte was the carrier of immune memory (Gowans, 1966), cellular theories of memory antibody responses invoked the linear differentiation of naïve lymphocytes into antigen-experienced cells (Sercarz, 1961; Makinodan, 1963; Sterzl, 1967; Celada, 1971). According to these models, re-encounter with antigen led to the terminal differentiation of antigen-experienced cells into antibody-secreting plasma cells. Importantly this focused attention on the changes induced in lymphocytes upon encounter with antigen that led to the propagation of memory.

It is now generally agreed that there are both phenotypic and functional differences between naïve and "antigen-experienced" or "memory" B and T lymphocytes. Despite this, it has been difficult to address the importance of these changes versus the simple increase in reactive cell frequency. Both of these processes are tightly connected with antigen priming during the primary response.

1.4 (b) Current issues in the study of immune memory

The two most important questions in immune memory are difficult to separate. Firstly, it is unclear what mechanism allows for the long-term maintenance of the memory state. Secondly, it is not known which of the phenotypic and functional changes that accompany the memory state are important for the heightened responses seen upon re-encounter with antigen.

Mechanisms for the persistence of the memory state

Various theories to account for the persistence of the memory state have been proposed and they can mostly be placed on a spectrum between the following two

extremes. On one side, memory results from the increased frequency of special antigen-specific B and T lymphocytes that carry enhanced reactivity toward antigen and are maintained in the absence of antigenic stimulation because of an intrinsically long lifespan. At the other end of the spectrum, memory simply reflects an increased frequency of antigen-specific lymphocytes that are constantly or intermittently stimulated to become effectors by persistent pathogens, reinfection, depots of processed antigens, bystander effects due to other immune responses or interaction with cross-reactive self-antigens. Elements of both of these theories appear to be true, as memory cells themselves do not seem to require contact with specific or cross-reactive antigen to be maintained, however continual antigen contact may be critically important for protective serum antibody production (Benner, 1981; Ahmed, 1996; Slifka, 1998a; McHeyzer-Williams, 1999). This notion may fit with the idea of the memory "stem cell" (Fearon, 2001). Under this definition, memory cells responsible for the maintenance of the memory state would have stem cell properties and self-renew by some mechanism, while presumably producing effector cells in an asymmetric division process like hematopoietic stem cells in the bone marrow.

A number of viral infections are either chronic (e.g. herpes viruses) or subject to repeated re-exposure (e.g. influenza), providing a potential mechanism for the intermittent restimulation of memory cells. However, memory to non-replicating antigens such as proteins also exists. In the late 1960's it was shown that small amounts of intact protein antigen could persist in lymphoid tissues for months after immunisation (Nossal, 1971; Tew, 1979). This was in the form of immune complex, which were hypothesised to allow for the continual stimulation of antigen-specific B (Tew, 1990) and helper T cells (Gray, 1991a) to maintain a state of memory. Subsequent cell transfer experiments by Gray and Skarvall (Gray, 1988) and Gray and Matzinger (Gray, 1991b) showed that both functional B and T cell memory declined rapidly with a half-life of around three weeks in the apparent absence of antigen. However, other studies estimated the lifespan of memory cells and concluded that only ~10 % of the memory B cell population is in active cell division at any one time (Schittek, 1990). This is difficult to reconcile with the cell transfer experiments and is more consistent with memory B cells being relatively quiescent and stimulated into cell division infrequently. One possibility is that Gray and Skarvall measured the decline of primed effector B cells by sampling the thoracic duct lymph, as many memory cells are in fact non-recirculating and found in sites such as the splenic marginal zone (Liu, 1988; Liu, 1991).

The view that contact with specific antigen is not required for memory cell persistence has received recent support. For example, B cell memory can be maintained in follicular dendritic cell-deficient mice (Karrer, 2000) and in the absence of T cell help

[Vieira, 1990 #18]. Also, in elegant recent experiments, Maruyama and colleagues used inducible gene targeting to switch the antigenic specificity of memory B cells in immunised mice to an antigen unrelated to the immunogen (Maruyama, 2000). They found that "memory" B cells expressing the new specificity were retained at constant levels for up to 9 months after the specificity switch. This suggests that, in the complete absence of specific antigen, memory B cells can persist for very long periods. The only caveat to this experiment is that the decay of these cells over time was not compared to naive B cells expressing the same anti-PE specificity. As the half-life of mature recirculating naïve B cells is of the order of months (Gray, 1988; Forster, 1990; Hao, 2001), the study should have ruled out the possibility that the new specificity had a long intrinsic lifespan unrelated to its priming status. A "News and Views" article describing this study is presented in Chapter 9 as an appendix. Finally, both $CD4^+$ and $CD8^+$ T cell memory has been shown to persist when memory T cells are transferred into MHC-deficient recipient mice (Murali-Krishna, 1999; Swain, 1999), suggesting T cell memory can persist in the absence of specific or cross-reactive antigen.

As well as the maintenance of memory cells, the memory state includes "protective immunity" in the form of elevated titres of antigen-specific antibody (Black, 1961; Benner, 1981; Simonsen, 1984; Ahmed, 1996). The half-life of bone marrow resident plasma cells is over 100 days (Manz, 1997; Slifka, 1998b). This places much less of a burden on the memory pool to constantly replenish plasma cells, and the finding that 10 % of memory B cells are engaged in cell division (Schittek, 1990) may allow sufficient differentiation to maintain the required numbers to sustain antibody production. In summary, while specific antigen persistence is not required for simple memory cell maintenance, it may be critically important for maintaining protective serum antibody levels. Much of the data concerning the maintenance of the memory state could then be reconciled by acknowledging the contributions of relatively quiescent "stem-cell like" memory cells as well as the continual low level production of long-lived plasma cells in response to persisting antigen.

Memory B cell phenotypes and locations

One of the phenotypic markers associated with a subset of memory B cells is the expression of switched isotype BCRs. This correlation was established by early cell transfer experiments demonstrating the enrichment of IgG^+ antigen-specific B cells in primed lymphocyte mixtures and the importance of those cells for transferrable memory (Black, 1977; Coffman, 1977; Yuan, 1977; Zan-Bar, 1977, Okomura, 1976 #253; Kanowith-Klein, 1979; Yefenof, 1985; Yefenof, 1986). For many years the expression of an isotype-switched BCR was the most unequivocal marker for memory B cells (Mackay,

1993), and has been used in this capacity in several recent experiments (Hayakawa, 1987; Maruyama, 2000). Studies on human peripheral blood B cells, however, have broadened the definition of memory B cells with respect to BCR isotype. The presence of IgM^+ IgD^- (Klein, 1997) and IgM^+ IgD^+ B cells (Klein, 1998) carrying somatic mutations and phenotypic markers such as CD27 shows that memory B cells do not have to carry switched isotype receptors. In rodents, some marginal zone-resident memory B cells also bear unswitched receptors (Liu, 1988), but most undergo switch recombination quickly during a secondary response (Toellner, 1996). The range of switched and non-switched isotypes expressed by somatically mutated B cells means the "gold standard" criterion for a memory B cell is now the expression of a somatically mutated BCR, showing signs of antigen-driven selection.

As well as BCR isotype, memory B cells have been ascribed other phenotypic markers, to the extent that there may be several phenotypic "subsets" of memory B cell, perhaps depending on their location. In humans, memory B cells have been described as $CD27^+$ (Klein, 1998; Tangye, 1998), $CD148^+$ (Tangye, 1998) and IgD^- $CD38^-$ $CD20^+$ $CD39^+$ $CD44^+$ (Bm5 tonsil subset) (Arpin, 1995; Liu, 1996a, Liu, 1995; Arpin, 1997). In addition human tonsil memory cells constitutively express CD80 and CD86 and can act as efficient APCs *in vitro* (Liu, 1995). A similar result has been reported for human peripheral blood memory B cells, where distinct $CD27^+$ $CD80^-$ and $CD27^+$ $CD80^+$ subsets have been identified (Bar-Or, 2001).

In the mouse memory B cells lose CD38 expression (Oliver, 1997b; Ridderstad, 1998). Interestingly, two recent studies in mouse have identified putative $B220^-$ memory B cells produced in response to immunisation with the hapten NP (McHeyzer-Williams, 2000; Driver, 2001). These have a $B220^-$, NP-binding, $CD11b^+$, IgG^+, $CD24^-$, $CD19^-$ and $CD22^-$ phenotype and may be functionally distinct from $B220^+$ memory B cells, forming plasma cells more rapidly upon *in vitro* culture.

The explosion of phenotypic definitions for memory B cells seems to reflect an underlying diversity in the memory B cell pool and may reflect a central theme in B and T cell memory. A similar diversity has recently been shown for memory T cells, with the description of $CCR7^+$ "central" memory and $CCR7^-$ "effector" memory cells that show distinct patterns of activation and cytokine production (Sallusto, 1999).

The most radical phenotypic distinction attributed to memory B cells comes from Klinman and colleagues who reported that memory B cell precursors in the mouse are enriched within the $J11D^{lo}$ ($CD24^{lo}$) pre-immune B cell repertoire (Linton, 1989). They speculated that memory B cells and primary antibody-forming cells form distinct lineages

(Ahmed, 1996; Klinman, 1997). Despite this, studies have shown a common clonal origin for plasma cells in extrafollicular splenic foci and germinal centre B cells, suggesting that a single B cell clone can seed both memory and AFC pathways (Jacob, 1992).

Memory B cells reside in a number of anatomical locations, including the blood (Klein, 1997; Klein, 1998; Bar-Or, 2001), the splenic marginal zone (Liu, 1988; Tierens, 1999) and similar areas in the tonsil (Liu, 1995) and Peyer's patch (Spencer, 1985). They have been termed "sessile" or non-recirculating (MacLennan, 2000) and seem to be absent from normal follicles (Kuppers, 1993). Despite this, Strober reported that thoracic duct lymphocytes passaged through a secondary host could adoptively transfer memory to a third animal (Strober, 1975) and Bachmann and colleagues showed that memory cells to VSV can move to other areas of the animal (Bachmann, 1994).

Mechanisms for the features of the memory response

A number of interconnected changes that accompany antigen priming have been used to explain the unique features of the memory response.

The simplest explanation for the rapid memory response is that antigen-specific B and T cells are maintained at elevated frequencies compared to the pre-immune repertoire (Ahmed, 1996; Doherty, 1996). For B cells, several studies have shown elevated frequencies of antigen-specific IgG^+ memory cells of between 0.01-0.07 % of total splenocytes under various immunisation regimes (Hayakawa, 1987; Schittek, 1990; McHeyzer-Williams, 1991; Smith, 1994; Kaisho, 1997; Maruyama, 2000). Early objections to the idea that memory could be carried by IgG^+ B cells (Zan-Bar, 1979) stemmed from their low frequency in normal animals where they seldom account for more than 1-2 % of lymphocytes (Gray, 1993). It was reasoned that large proportions of IgG^+ B cells should accumulate over the lifetime of an animal if they were solely responsible for memory. To a large extent this problem has been solved by the observations that a large fraction of human peripheral blood B cells are non-switched memory cells (Klein, 1997; Klein, 1998) and many non-switched marginal zone memory cells rapidly switch isotype during a secondary response in mice (Toellner, 1996). It has also been proposed that memory B cells are poised to rapidly produce plasma cells and so are biased towards terminal differentiation, a mechanism that might stop "repertoire freezing" due to the accumulation of too many memory cells (Arpin, 1997). This finding may also explain the dominance of extrafollicular focus reactions over germinal centre formation during the secondary response, as memory cells are poised to differentiate into plasma cells rather than seed GCs (Liu, 1991; Arpin, 1997; Vora, 1998).

As well as increased frequency, other phenotypic and functional changes play a role in the rapid initiation of memory responses. As discussed earlier, memory B cells localise to antigen-draining sites and are rapidly mobilised to migrate to the outer PALS upon antigen binding (Liu, 1991). This, coupled with the ability to drive the secondary response as APCs, by virtue of high affinity antigen receptors and the rapid up-regulate co-stimulatory molecules (Liu, 1995; Bar-Or, 2001), means memory B cells play a more active role in initiating the secondary response. This may be one reason why immuno-stimulatory adjuvant is not usually required for the induction of a memory response (Liu, 1991; McHeyzer-Williams, 1991; Kaisho, 1997). In the same way, memory T helper cells show a number of phenotypic changes, including more efficient activation and cytokine secretion as well as a faster rate of cell division (Rogers, 2000).

The rapid initiation of memory responses is easy to envisage, given the qualitative and frequency differences of memory cells versus naïve cells. However it is not so obvious how multiple $\log_{10}$ increases in the amount of antibody can be produced during the secondary response, especially in light of recent evidence that there are extrinsic factors in the spleen that limit the survival of plasma cells (Sze, 2000). Also the effective antigen dose driving the response should be lower, due to the presence of high affinity neutralising antibody from the primary response and the rapid production of high affinity antibody from secondary plasma cells.

The dominance of isotype-switched antibody during TD secondary responses to protein and hapten antigens argues that memory B cells bearing isotype-switched receptors (Okomura, 1976; Hayakawa, 1987; McHeyzer-Williams, 1991), or memory B cells that switch rapidly after stimulation (Toellner, 1996) are a major responding B cell population. Certainly the expression of isotype-switched antigen receptors has been used as a marker for memory B cells, and the active role of these cells in memory is in keeping with numerous early cell transfer experiments where adoptively transferrable memory could be depleted from primed lymphocyte mixtures by removal of IgG^+ cells. In fact, isotype switching from IgM and IgD to downstream isotypes produces profound changes in the structure of the BCR within the membrane, and several studies have documented differences in signal transduction through switched receptors, both *in vitro* and *in vivo*. At this stage, however, it is unknown whether signalling through isotype-switched BCRs influences the TD memory response *in vivo*.

The aim of this thesis is to test whether switched BCR isotypes play a role in B cell responses to antigen *in vivo*, in isolation of the other factors induced by antigen priming. The next section will detail *in vitro* and *in vivo* evidence that switched BCR isotypes have distinct signalling properties and will outline the experimental design of the current study.

Section 1.5. Evidence for isotype-specific BCR signalling *in vitro* and *in vivo*

The close association of isotype switching with memory B cells and the dominance of switched isotypes, especially IgG, in memory antibody responses is indirect evidence that BCR isotype may play a role in the responses of mature B cells to antigen. This is a correlation only, and isotype switching may simply provide a method of diversifying the effector function of secreted antibodies, with little impact on signal transduction *via* the BCR. The following sections will review various lines of evidence that show that isotype-specific BCR signalling may influence cellular responses in both naïve and memory B cells.

1.5 (a) BCR isotypes differ in their structure within the membrane

Membrane-bound immunoglobulin of all isotypes is produced *via* alternative splicing of Ig heavy chain exons to exclude short secretory segments and include two isotype-specific 3' exons encoding the extracellular spacer, transmembrane and cytoplasmic sections that make up the membrane tail. The constant regions of mouse heavy chain isotypes vary, with either 4 (IgM, IgE), 3 (IgG, IgA) or 2 (IgD) C_H domains. This gives different degrees of flexibility to these molecules, with IgM and IgE relatively restricted by small hinge regions and IgD and some IgG isotypes relatively mobile with extensive hinge regions. The isotype-specific membrane tails provide further variation, especially in the extracellular spacer and cytoplasmic sections (Reth, 1992).

All isotypes share a similar predicted transmembrane domain, with 13 out of 25 amino acids conserved across isotypes (see Figure 1-3). The most significant differences at this point include a conserved cysteine in mouse and human IgG isotypes and a proline in mouse IgD and mouse and human IgA. A number of hydrophilic residues are present in this domain, suggesting protein/protein interactions are required to mask these from the lipid bilayer. Amphipathic analyses of the predicted membrane-spanning alpha helix reveal that most of the conserved residues are located on one face of the molecule, possibly at a common contact point for the Igαβ heterodimer (Reth, 1992). The other face may be involved in isotype-specific contacts.

By contrast, the extracellular spacer and cytoplasmic tail sequences differ extensively between isotypes, but are highly conserved between isotype subclasses in the same

organism and between isotypes in different organisms. The extracellular spacers are rich in charged amino acids and vary in length between isotypes, for example, extracellular spacer regions in human isotypes vary from 13 aa (IgM) to 66 aa (IgE_{long} isoform). In addition, interchain disulfide bonds have been shown to form between conserved cysteine residues in the extracellular spacers of human IgG, IgA and IgE membrane isotypes (Bestagno, 2001), possibly playing a stabilising role. The conserved cytoplasmic sequences of membrane Ig isotypes also show large variation between isotypes. The predicted cytoplasmic sequence for IgM and IgD is just 3 aa long, while IgG and IgE have 28 aa tails and IgA a 14 aa tail (Reth, 1992), although this is subject to uncertainty and may depend on the techniques used to predict the membrane-spanning regions (Klein, 1985).

The high degree of conservation of these membrane tail regions within isotypes and between species suggests some evolutionarily conserved function of these sequences. It has been speculated that variation in the extracellular spacer regions leads to the association of various isotypes with differentially glycosylated forms of Igαβ (Campbell, 1991; Pogue, 1994), partly controls the strength of association with Igαβ (Schamel, 2000), and may play a role in isotype-specific association with BCR-associated proteins (BAPs) (Batista, 1996). In addition, the extended cytoplasmic sequences found in IgG and IgE have been implicated in providing a partial endocytosis signal (Weiser, 1994; Achatz, 1997; Kaisho, 1997; Knight, 1997; Nussenzweig, 1997). Experiments to determine isotype-specific BCR signalling functions will be outlined below.

1.5 (b) Attempts to delineate isotype-specific signalling functions

Two approaches have been taken to study isotype-specific BCR signalling. One explores the details of BCR biochemistry, where signalling *via* the various isotypes is probed *in vitro*. The second approach relies on the ability of immunoglobulin transgenic and knockout mice to reveal similarities and differences between the behaviour of B cells expressing various BCR isotypes *in vivo*.

In vitro studies

All of the immunoglobulin subtypes associate with Igα/β (Venkitaraman, 1991; Batista, 1996; Leduc, 1997), however under some circumstances both IgG and IgD can be expressed on the cell surface in the absence of the Igα/β heterodimer.

While IgG seems to associate with Igα/β if the complex is present, studies have shown that IgG_1 (Knight, 1997), IgG_{2a} (Weiser, 1994) and IgG_{2b} (Venkitaraman, 1991) can

all be expressed at the surface of transfected cells in the absence of Igα/β. Also, IgD can be expressed on the surface of the Igα/β negative cell line J558L as a GPI-linked protein (Weinands, 1992). The implications of IgG and IgD expression without the Ig α/β complex for signal transduction in primary B cells is obscure as it is not known to what extent these Igα/β-independent mechanisms lead to IgG and IgD expression *in vivo*.

Isotype-specific signalling has been studied most extensively in comparisons of IgM and IgD isotype BCRs. Developmentally-regulated changes in the expression of these isotypes suggests they may have distinct signalling functions. The best evidence that IgM and IgD transduce different signals comes from studies in immature B cell lines, showing that IgM crosslinking led to growth inhibition while IgD crosslinking had no effect (Ales-Martinez, 1988; Tisch, 1988). At present it is not clear what signals might explain these differences, although IgM and IgD have been shown to associate with differently glycosylated forms of the Igα/β heterodimer (Campbell, 1991; Venkitaraman, 1991; Pogue, 1994). Also, IgM and IgD associate with different intracellular proteins, termed BCR-associated proteins (BAPs) (Kim, 1994; Terashima, 1994). IgM interacts with BAP 32/prohibitin, BAP 37 and BAP 41, while IgD interacts with BAP 29 and BAP 31. These proteins are much more ubiquitously expressed than the Igα/β heterodimer and may play general roles, as regulators of proliferation and mitochondrial function (BAP 32/prohibitin and BAP 37) (Wang, 1999; Coates, 2001) or of protein trafficking (BAP 31) (Annaert, 1997).

Given the high degree of sequence conservation in the membrane tail domains of isotype-switched BCRs, it is surprising that very few signalling pathways or interacting proteins unique for isotype-switched antigen receptors have been described. In terms of early signalling events, one report suggested that IgG transfectants of a murine lymphoma line were more sensitive to stimulation at the level of calcium mobilisation compared to IgM transfectants (Leung, 1994). The induction of tyrosine phosphorylation after receptor cross-linking has also been shown to be stronger in some human follicular lymphoma cell lines expressing IgG compared to IgM (Eray, 1998). However, another study using chimeric CD8α/γ2a molecules concluded that the IgG2a cytoplasmic tail sequences alone could not induce tyrosine phosphorylation after crosslinking (Kim, 1993). In agreement with this, Pogue found that primary B cells from anti-HEL transgenic mice expressing an IgG_1 isotype BCR (Pogue, 2000) showed a profound defect in tyrosine phosphorylation induction *in vitro* compared to IgM transgenic cells (Pogue, 1996). Despite this, the calcium flux response in the anti-HEL IgG transgenic cells was more robust than the IgM transgenic cells suggesting the IgG and IgM receptors transduce qualitatively different signals.

Figure 1-3. Sequence comparison of membrane Ig isotypes from various species at the extracellular, membrane-spanning and cytoplasmic regions

Sequence information was taken from a review by Reth (Reth, 1992). Asterisks denote the conserved residues in the putative transmembrane domain. Dashes show sequence identity with the uppermost sequence, membrane-bound mouse IgM. Note the extended predicted cytoplasmic sequences of isotype-switched IgG, IgE and IgA isotypes in comparison to IgM and IgD. Also, note that the predicted extracellular membrane-proximal domains of each isotype share minimal sequence identity.

Membrane-bound Ig isotypes - variation at the level of the membrane tail domain

Extracellular	Transmembrane (predicted)	Cytoplasmic	
	** * * ***** ** **		
EGEVNAEEEGFEN	LWTTASTFIVLFLLSLFYSTTVTLP	KVK	mouse IgM
------------	-------------------------	---	human IgM
---G---------	-------------------------	---	rabbit IgM
QHSCIMDEQSDSYMDL-EENG	--P-MC--VA----T-L--GF--FI	---	mouse IgD
LIPQSKDENSDDYTTFDDVGW	----L---VA--I-T-L--GI--FI	---	human IgD
GLQLD-TCAE-QDGELDG	----ITI--S-----VC--AA----	---WIFSSVVELKQTLVPEYLNMIGQAP	mouse IgG1
ELELN-TCAE-QDGELDG	----ITI--S-----VC--AS----	---WIFSSVVQVKQTAIPDYRNMIGQGA	mouse IgG3
ELQLE-SCAE-QDGILDG	----ITIL-T-----VC--A---F-	---WIFSSVVDLKQTIIPDYRNMIGQGA	human IgG3
ELDLQDLLCIEEV-G-EL-E	---SICV--T-----VS-GA---VL	---WVLSTPMQDTPQTFQDYANILQTRA	mouse IgE
SYVLLDQSQDILE--APGAS	--P-TV--LT----------AL-VT	T-RGPFGSKEVPQY	mouse IgA
PYVVLDLPQETLE--TPGAN	--P-TI--LT----------AL-VT	S-RGPSGNREGPQY	human IgA1

Potential differences in downstream signalling targets have also been seen between isotype-switched and naïve BCRs. For example, IgG but not IgM isotype antigen receptors on tonsillar B cells or EBV-transformed B cell lines were able to activate an NADPH-dependent oxidase (Leca, 1991) complex in a Rac-dependent manner (Dorseuil, 1992). More recently, a B cell specific DNA recombination complex called SWAP has been described in the mouse, consisting of nucleophosmin, nucleolin, poly(ADP-ribose)polymerase and SWAP-70 (Borggrefe, 1998). Interestingly, the human homologue of SWAP is expressed in mature B cells and contains nuclear import, export and membrane-localising motifs. SWAP-70 seems to be located in the cytoplasm in resting B cells, however upon activation *in vitro* with either LPS or CD40/IL-4 it first associates with the plasma membrane then translocates to the nucleus and finally co-localises and can be co-precipitated with IgG, but not IgM (Masat, 2000). When SWAP-70 is disrupted in knockout mice, B cells show higher radiation sensitivity and impaired switching to some downstream isotypes, although the role of SWAP-70 in those processes is not clear (Borggrefe, 2001). Finally, a connection between isotype-switched BCR signalling and CD30/CD30L (CD153) signalling has been suggested. It was found that CD30L signalling by receptor-crosslinking or interaction with $CD30^+$ T cells in human IgM^+IgD^+ B cells lead to the suppression of isotype switching and terminal B cell differentiation (Cerutti, 2000). IgG^+ tonsillar B cells were not inhibited by this interaction, suggesting the IgG isotype BCR transduces signals that are qualitatively different to IgM and IgD and can compensate for CD153 inhibitory signalling.

Some proteins have been shown to associate specifically with isotype-switched receptors. Pogue found three phosphoproteins in M12 lymphoma transfectants that associated preferentially with the cytoplasmic tail region of IgG_1 (Pogue, 1996). These were described as proteins of 20, 21 and 45 kDa that associated exclusively with intracellular immunoglobulin after immunoprecipitation from transfected cell lysates. Also, in a comparison of two human IgE BCR isoforms expressed in WEHI-231 cells, Batista *et al.* found that both the long and short IgE isoforms but not the endogenous IgM BCR associated with proteins of 37 kDa and 41 kDa (Batista, 1996).

The final isotype-specific function that has been ascribed to isotype-switched receptors is in antigen presentation, involving the targeting of antigen/BCR complexes into the endocytic pathway for antigen degradation and loading onto MHC Class II molecules (Siemasko, 2001). Although IgM and IgD can both act as antigen presentation modules through signals in their heavy chains and associated Igα/β heterodimers, the YXXM motif found in the cytoplasmic tail domain of the IgG and IgE subtypes has been proposed to act as an additional antigen presentation motif (Achatz, 1997; Kaisho, 1997; Knight, 1997;

Nussenzweig, 1997). YXXΦ motifs (where Φ is a bulky, hydrophobic amino acid) has been shown to be a signal for the recruitment of the μ2 component of the AP-2 complex involved in the clathrin-mediated endocytosis (Kirchausen, 1997) of receptors such as CTLA-4 (Shiratori, 1997). It has been observed that a NIP-specific IgG_{2a} BCR, expressed on the cell surface of J558L cells in the absence of Igα/β could be efficiently internalised after the addition of antigen (Weiser, 1994). Interestingly an earlier study found that the cytoplasmic tail domain of IgG_{2b} alone could not rescue the presentation of antigen when expressed as a chimera with IgM in the absence of Igα/β (Patel, 1993), suggesting multiple signals in addition to the tail domain may be required for antigen presentation. As an extension to these findings, a tetanus toxin-specific IgG_1 BCR could be expressed in an Igα/β-negative cell line yet still mediate antigen presentation to T cells, an event partially dependent on the cytoplasmic tail domain (Knight, 1997). The authors take this as evidence of an antigen-targeting motif in the IgG_1 cytoplasmic tail domain, although antigen presentation *via* IgG_1 in the absence of Igα/β is only partially reduced by deletion of the tail. The identification of this putative antigen-presentation signal in the cytoplasmic tail domain of IgG has been proposed to account for the decreased secondary antibody responses in mice with truncations of the cytoplasmic tail domains of IgG_1 (Kaisho, 1997) and IgE (Achatz, 1997), although there are alternative explanations for these *in vivo* studies that will be discussed later.

In vivo studies

Despite *in vitro* data that suggests there are signalling differences between IgM, IgD and isotype-switched receptors, the evidence for different biological functions between BCR isotypes *in vivo* from knockout and immunoglobulin transgenic models is scarce.

Knockout studies have attempted to delineate the roles of IgM and IgD in the development and functional maturation of B cells by disruption of one or other isotype by gene targeting. IgM seems capable of providing signals for the production of fully mature peripheral B cells capable of responding to T-dependent and T-independent antigens in the absence of IgD (Roes, 1991, 1993). The only noticeable effects of IgD deficiency was a selective disadvantage of IgD^- cells in mice heterozygous for the mutation, as well as a slightly delayed rate of affinity maturation during the primary immune response to a T-dependent antigen. As well as IgM, IgD is also capable of promoting the production of fully mature peripheral B cells (Lutz, 1998). In a selective $IgM^{-/-}$ mouse that continued to express IgD, B cell development through to mature B cells was normal, and IgD provided normal functions for antibody secretion, isotype switching, responses to T-independent type 2 and T-dependent antigens, and germinal centre formation. The only defects

observed were in lower numbers of $B220^{+}CD5^{+}$ B-1a peritoneal B cells and a delayed onset of neutralising antibody production to the cytopathic vesicular stomatitis virus.

These knockout studies mirror results from immunoglobulin transgenic mice in which rearranged IgM or IgD (or both) transgenes are inserted into the mouse genome (Goodnow, 1992). Both IgM and IgD transgenes are capable of mediating endogenous heavy chain allelic exclusion (Weaver, 1985; Iglesias, 1987; Nussenzweig, 1987) and the formation of mature peripheral B cells. One early hypothesis claimed that the developmentally regulated expression of IgD inteferes with peripheral tolerance and that once IgD is expressed, B cells make proliferative rather than apoptotic responses (Carsetti, 1993) upon antigen exposure. This interpretation is unlikely for two reasons. Firstly it has been shown that signalling through either IgM or IgD leads to apoptosis in immature splenic B cells but not in mature splenic B cells (Norvell, 1996). Secondly IgM-only transgenic lines show no defects in response to T-dependent antigens, indicating that IgM can transmit activation signals (Brink, 1992). In fact it seems that apoptotic responses by peripheral B cells correlate with B cell maturation state rather than BCR isotype.

A limited number of knockout and transgenic studies have also investigated whether isotype-switched receptors can allow normal B cell development. The role of IgG in promoting allelic exclusion and B cell development has been studied extensively with IgG transgenic mice (Yamamura, 1986; Tsang, 1988; Offen, 1992; Tsao, 1992; Roth, 1993; Iliev, 1994; Kenny, 1995; Roth, 1995; Battegay, 1996; Kurtz, 1996; Pogue, 2000). The results have been confusing. While some studies have demonstrated IgG-mediated allelic exclusion of endogenous IgM expression in the bone marrow (Roth, 1993; Iliev, 1994; Kenny, 1995; Battegay, 1996), others have seen no evidence of allelic exclusion (Yamamura, 1986; Tsao, 1992). In addition, the ability of transgenic IgG expression alone to allow normal B cell development has been observed in some cases (Offen, 1992; Iliev, 1994; Kenny, 1995) and not in others (Roth, 1993; Roth, 1995; Battegay, 1996). Recently Pogue and Goodnow produced a panel of anti-lysozyme IgG_1 transgenic lines and provided a hypothesis that may explain many of the disparate finding surrounding IgG transgenics (Pogue, 2000).

The transgenic lines were made using the same V_H/V_L anti-HEL combination described previously (Goodnow, 1988). In this case the heavy chain constant regions were either from IgG_1 or from a chimeric heavy chain (IgM/G) consisting of the IgM C_H exons and the IgG_1 M1 and M2 exons which encode the extracellular spacer, transmembrane and cytoplasmic sequences of the membrane tail of IgG_1. It was noticed that a variable number of HEL-binding B cells were present in the peripheral compartments of the different lines, and this correlated with the transgene copy number integrated in the mouse genome, so that

lines with more copies produced fewer peripheral B cells expressing the transgenic receptor. This observation was supported in spontaneous copy-loss variants derived from a high copy number IgM/G transgenic line where a clear increase in the proportion of mature HEL-binding lymphocytes was observed in both copy-loss variants (18 % and 52 %) compared to the parental line (6 %). The number of transgene copies correlated also with the level of HEL-binding receptor expressed on pro-B cells in the bone marrow of these mice. The hypothesis to explain this was that higher IgG heavy chain expression early in development leads to receptor editing away from the expression of the transgenic heavy chain and a subsequent decrease in the number of mature HEL-binding B cells in the periphery.

Importantly, these observations may help explain variable results gained from a panel of $IgG2_b$ transgenics carrying heavy chain genes from a *Pseudomonas aeruginosa* -specific antibody. Two of the transgenic lines from this panel (343-1, 348A) showed no evidence of normal mature $IgG2_b^+$ peripheral B cells (Roth, 1993), whereas one "variant" line from this panel showed both allelic exclusion and normal production of $IgG2_b^+$ peripheral B cells (343C). Both the 343-1 and 348A lines had relatively high transgene copy numbers (343-1, 8 copies; 348A, 22 copies) whereas the variant 348C line had a lower transgene copy number (3 copies) (Roth, 1995). This line was derived in a similar way to the anti-HEL copy-loss variants as the by-product of a very high copy number parental line (348-4-8, 90 copies) (Tsang, 1988).

In vivo *studies of isotype-switched B cell antigen receptors*

Only two studies, using identical approaches, have studied the role of isotype-switched receptors in B cell responses to antigen *in vivo*.

Targeted gene-knockout studies of IgG_1 (Kaisho, 1997) and IgE (Achatz, 1997) demonstrated the importance of the membrane expression of these isotypes for proper IgG_1 and IgE antibody responses. It is not surprising that the complete ablation of membrane IgG_1 or membrane IgE expression led to drastically decreased serum titres in pre-immune and immunised animals, given the requirement of BCR expression for peripheral B cell maintenance (Lam, 1997). More interestingly, knockouts in which only the extended cytoplasmic tail sequences of IgG_1 and IgE were removed (Δtail mice) showed an intermediate defect in serum titres and immune reactivity during primary and secondary antibody responses.

In their study of IgG_1, Kaisho *et al.* speculated that the IgG_1 cytoplasmic tail sequence acted to increase antigen presentation to helper T cells, on the basis of *in vitro*

work suggesting an antigen presentation role for the IgG_1 cytoplasmic tail sequences (Knight, 1997). Despite this hypothesis, the cellular mechanism behind the action of the IgG_1 cytoplasmic sequence was not actually established in this study. For example, while Kaisho *et al.* showed that fewer antigen-specific IgG_1^+ cells accumulated in Δtail mice after a primary T-dependent antibody response, the reason for this could not be determined. Potentially, the IgG tail domain could have allowed more efficient cell activation, a greater rate of cell division, protection from cell death, or more efficient germinal centre formation.

More seriously, the experimental strategy employed by Kaisho *et al.* relied on the expression of a truncated form of the IgG_1 BCR. This mutant BCR lacked evolutionarily conserved membrane-proximal motifs, and was made with no prior knowledge of the effect of this modification on membrane insertion or the stability of cell surface expression. In fact there is compelling evidence that the expression of the Δtail IgG BCR was decreased compared to IgG on normal wild-type B cells after stimulation with LPS and IL-4 *in vitro* (Kaisho, 1997). In addition, there was a greatly decreased IgG_1 serum antibody titre and many fewer IgG_1^+ B cells, even in pre-immune Δtail mice. This raises an alternative interpretation for this study: namely that poor immune responses in Δtail mice are not due to the disruption of a motif required for antigen presentation or cell activation, but that the inefficient surface expression of a truncated, mutant IgG BCR is not permissive for B cell survival.

In summary, the role of isotype-switched BCR expression in B cell responses to antigen *in vivo* remains an open question. The studies of Kaisho (Kaisho, 1997) and Achatz (Achatz, 1997), while showing the importance of the IgG and IgE cytoplasmic tail regions for B cell biology have not determined whether those sequences are involved in stable membrane expression or some signalling function associated with cell activation or antigen presentation. If their studies do reflect some isotype-specific signalling function for those regions, they have not established a cellular mechanism for their action, which may range from modulating cell activation, cell division, death or differentiation rates, to some aspect of affinity maturation.

Section 1.6. The current study

There have been very few experiments to directly test the role of isotype-switched BCRs in memory responses. As discussed above, this is due to the host of changes that accompany antigen-induced priming, and the lack of a strategy to separate these has meant

that the specific role of isotype-switched signalling in memory responses has not been determined.

In order to fully address the role of isotype-switched receptors and their unique membrane tail domains in B cell responses to antigen *in vivo*, there are several requirements that must be met by an experimental system.

(1) Comparisons should be made between B cells bearing IgM versus IgG (or other isotype-switched BCRs) that share identical antigen specificity and affinity.

(2) B cells from the comparison groups must be matched in their maturation stage, antigen experience and precursor frequency.

(3) To test the role of the unique IgG membrane tail (for example), the "add-back" experiment should be performed, where the IgG membrane tail is added to IgM. This directly tests the ability of that segment to confer novel functions to IgM, and avoids the difficulty in interpreting loss of function phenotypes produced by deleting or mutating the membrane tail sequence.

To address these requirements and isolate the role of BCR isotype in B cell responses to antigen *in vivo*, this study has compared populations of naïve B cells from anti-Hen Egg Lysozyme (HEL) immunoglobulin (Ig) transgenic mice that express uniformly high-affinity BCRs of a defined isotype (Goodnow, 1988; Brink, 1992; Pogue, 1994; Pogue, 1996; Pogue, 2000) (see Figure 1-4). In the different mouse lines, the constant region segments are IgM or IgD (naïve BCR isotypes), or are IgG (isotype-switched BCR). The IgG expressing lines contain either the full IgG constant region, or a chimeric IgM/G constant region where the constant region exons of the secreted product are from IgM but the two membrane exons encoding the membrane tail are from IgG (see Figure 2-1). B cells from these transgenic lines were seeded into T-dependent immune responses *in vivo*, and the outcome in terms of clonal expansion and differentiation tracked at the single cell level.

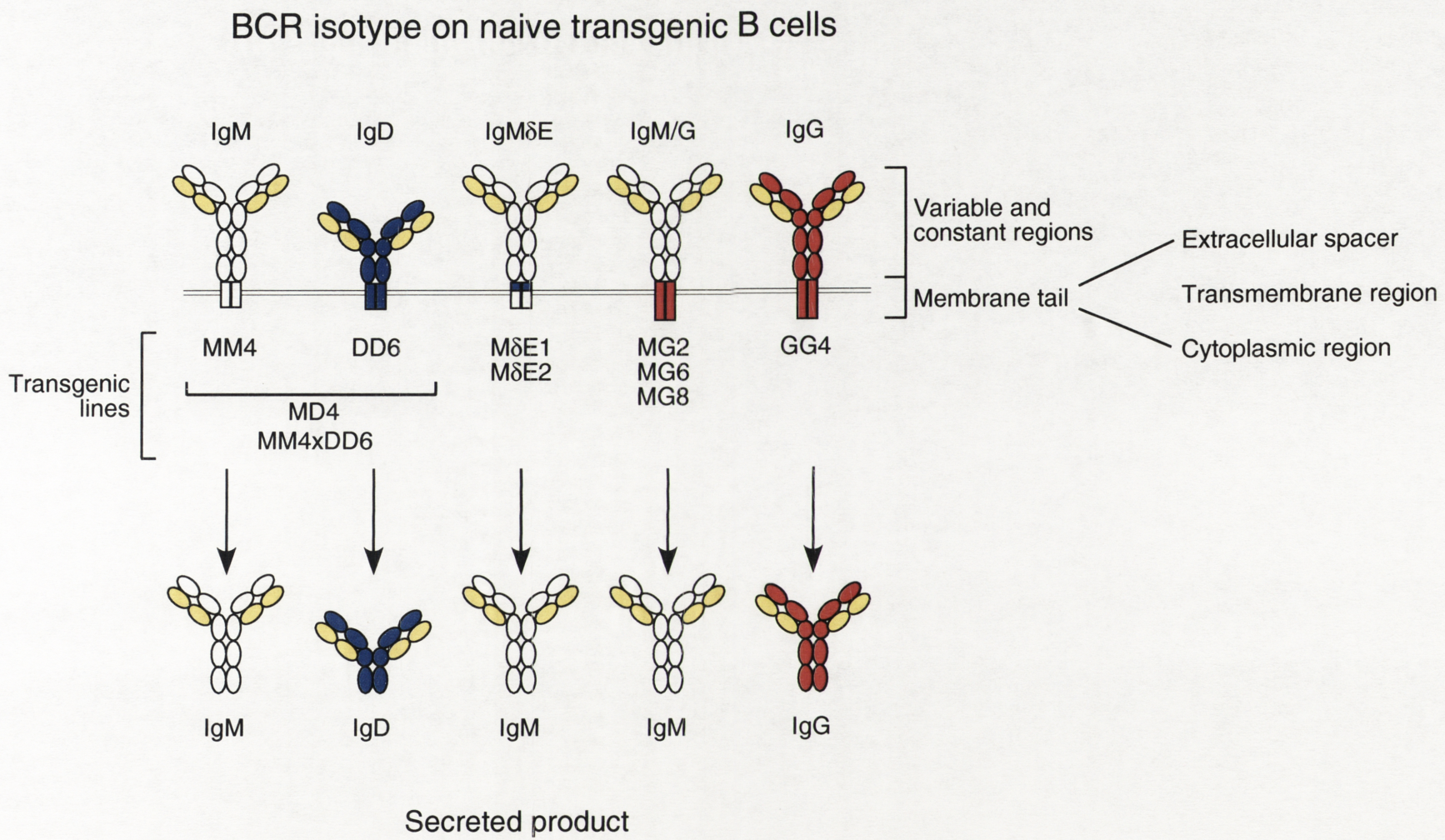
This experimental design is able to control for many of the variables not accounted for by earlier work. Firstly, the panel of Ig transgenic mice share identical variable regions with a uniform high affinity for HEL. This ensures homogeneous populations of B cells are compared that share the same specificity and affinity for a specific antigen. Secondly, the transgenic constructs retain important conserved domains, such as the entire extracellular spacer, transmembrane and cytoplasmic sequences of IgG_1 and IgM. This ensures that membrane expression in these lines is equivalent, which is clear from an analysis of immature bone marrow B cells (Pogue, 2000). This removes the possibility that differences in responsiveness between lines is due to inefficient surface BCR expression.

Most importantly, the issue of antigenic experience is controlled by the fact that all transgenic B cells are naïve, having never encountered HEL prior to adoptive transfer. This removes the host of other phenotypic changes that accompany memory cell formation as a potential complicating factor in these experiments. Finally, adoptive transfer allows the number of antigen-specific B cells to be systematically varied. This means the precursor frequency of high affinity antigen-specific cells of a defined isotype can be controlled, and valid comparisons made between B cells carrying IgM or IgG isotype receptors that are present in equal numbers.

This thesis describes experiments using this system to investigate the question of the role of BCR isotype in B cell responses to a T-dependent antigen *in vivo*. This study will present evidence to show that the IgG transgenic B cells make more robust responses to antigen compared to B cells expressing IgM or IgD, and that the IgG membrane tail is the key molecular regulator of this response. The IgG membrane tail acts to protect B cells from cell death over successive cell divisions, resulting in a much greater clonal burst size and more antibody-forming cells compared to B cells expressing IgM and IgD. This establishes the expression of isotype-switched BCRs as a key part of "priming" the immune system to make more robust effector responses on re-exposure to T-dependent antigen.

Figure 1-4. Structure of the BCR isotypes from the various transgenic strains used in this study

The various forms of BCR isotype used by each anti-HEL immunoglobulin transgenic line used in this study are shown. IgM isotype heavy chain is shown in outline. IgD heavy chain is shown in blue. Note the extracellular membrane-proximal section of IgD in the MδE lines. IgG_1 heavy chain is shown in red. Note the extracellular membrane-proximal, transmembrane and cytoplasmic sequences of the IgG_1 membrane tail in the MG lines. Light chains are shown in yellow. Each line shares an identical high-affinity HEL-specific heavy and light chain variable region derived from the anti-HEL hybridoma HyHEL10 (Smith-Gill, 1982; Smith-Gill, 1984a; Smith-Gill, 1984b; Goodnow, 1988). The secreted antibody product produced by plasma cells from each line is also indicated.


BCR isotype on naive transgenic B cells
IgM
IgD
IgMδE
IgM/G
IgG
Variable and constant regions
Membrane tail
Extracellular spacer
Transmembrane region
Cytoplasmic region
Transgenic lines
MM4
DD6
MδE1
MδE2
MG2
MG6
MG8
GG4
MD4
MM4xDD6
IgM
IgD
IgM
IgM
IgG
Secreted product

Chapter 2. Materials and Methods

Section 2.1. Materials

All chemicals used in the production of buffers and stock solutions were AR grade or better. The source of specialised reagents and their grade has been indicated throughout the materials and methods section where appropriate.

Section 2.2. Mouse lines (description and housing)

All mice were housed under SPF conditions within the ACRF Medical Genome Centre at the John Curtin School of Medical Research, Australian National University, Canberra.

Immunoglobulin (Ig) transgenic mice were strains MD4 (Goodnow, 1988); MM4 (Brink, 1992); GG4, MG2, MG6 and MG8 (Pogue, 2000); MδE1 and MδE2 (Pogue, 1994); DD6 (Brink, 1992) and (MM4xDD6)F_1. Ig transgene construct information and the sequence of the membrane tail regions encoded by exons M1 and M2 for each Ig transgenic strain is given in Figure 2-1. The TCR transgenic strain was 3A9 (Ho, 1994).

Ig transgenic lines were maintained on the C57BL/6, C57BL/6:RAG1$^{-/-}$, (B10.BRxC57BL/6)F_1, (B10.BRxC57BL/6)F_1:RAG$^{-/-}$ and B10.BR:RAG1$^{-/-}$ backgrounds. TCR transgenic lines were maintained on the (B10.BRxC57BL/6)F_1, B10.BR and B10.BR:*FasL*^{gld} backgrounds.

Recipient mice for adoptive transfer experiments were either C57BL/6, B10.BR or (B10.BRxC57BL/6)F_1 hybrids. No differences were noticed when male or female mice were used as recipients. Mice were used as recipients between 8 and 15 weeks of age.

Section 2.3. Transgenic mouse screening

TCR and immunoglobulin transgenic mice were screened by PCR analysis of ear punch DNA and by flow cytometric analysis of blood. PCR analysis of ear punch DNA was also used to screen the H-2 haplotypes of (MM4:H-2^{kb} x DD6:H-2^{bb})F_1 mice.

2.3 (a) PCR screening

Screening of mice by ear punch DNA and PCR was performed according to the method of Chen and Evans (Chen, 1990). Briefly, ear punches were collected in eppendorf tubes during the routine marking of mice. These punches were digested in 20 µl buffer

containing 50 mM Tris.HCl (pH 8.0, Boehringer Mannheim, Indianapolis, IN), 2 mM NaCl, 10 mM EDTA, 1 %(w/v) SDS (Bio-Rad, Hercules, CA) and 1 mg/ml Proteinase K (Roche Molecular Biochemicals, Indianapolis, IN). Digestion was for 40 minutes at 55 °C with vigourous vortexing after 20 minutes. ddH_20 was added to a final volume of 200 µl and the samples boiled at 100 °C for 5 minutes to destroy Proteinase K activity. Digested samples were stored at -20 °C until PCR analysis.

PCR reactions were performed in a 50 µl volume containing 1 µl of digested ear punch, 5 µl 10x PCR buffer (where 1x = 67 mM Tris.HCl pH 8.8, 16 mM ammonium sulfate, 10 mM 2-mercaptoethanol and 2.5 mM magnesium chloride), 1 µl dNTPs (10 mM with respect to dATP, dCTP, dGTP and dTTP, Amersham Pharmacia Biotech, Uppsala, Sweden), 0.5 µl TX-100 (molecular biology grade, Sigma Chemical Co., St Louis, MO), oligonucleotide primers (BRF facility, JCSMR), 0.25 µl Taq polymerase (prepared in house) and sufficient ddH_20 for 50 µl total volume. 2-mercaptoethanol was molecular biology grade from Sigma.

Oligonucleotide primer 5' → 3' sequences and the amounts per reaction for each PCR screening protocol are given below:

(1) *Immunoglobulin transgenic*:

IgH-F_1	GCGACTCCATCACCAGCGAT	100 ng
IgH-F_2	CTGGAGCCCTAGCCAAGGAT	25 ng
IgH-R_1	ACCACAGACCAGCAGGCAGA	25 ng

(2) *TCR transgenic*:

TCR-F_1	GCAGTCACCCAAAGCCCAAG	150 ng
TCR-R_1	CCCCAGCTCACCTAACACTG	150 ng
IgH-F_1	GCGACTCCATCACCAGCGAT	100 ng
IgH-R_1	ACCACAGACCAGCAGGCAGA	25 ng

(3) *H-2 haplotype*:

Eα5'	AGTCTTCCCAGCCTTCACACTCAGAGGTAC	150 ng
Eα3'	CATAGCCCCAAATGTCTGACCTCTGGAGAG	150 ng
K5'	CATGGGCATAGAAAGGGCACTCTTTGAACT	150 ng

All PCR reactions were carried out in a Corbett Research FTS-960 Thermal Cycler using 35 cycles of 15 s denaturing at 94 °C, 30 s annealing at 60 °C and 45 s elongation at 72 °C. 20 µl of PCR reaction products were run on a 2 % (w/v) agarose electrophoresis gel with incorporated ethidium bromide, and the products photographed under UV light.

Immunoglobulin transgenic PCR yielded a 430 bp immunoglobulin transgene band and a 264 bp endogenous immunoglobulin band. TCR transgenic PCR yielded a 371 bp TCR transgene band and a 264 bp endogenous immunoglobulin band. H-2 PCR yielded a 150 bp H-2^b haplotype band and a 250 bp H-2^k haplotype band.

2.3 (b) Blood screening

For screening by blood, a small volume of tail vein blood was taken using a 26 gauge needle and a heparinised capillary tube (Hirschmann Laborgerate, Germany). Blood was blown into 100 µl Alsever's solution (prepared in house). After 2 rounds of erythrocyte lysis for 10 minutes at room temperature using Tris-Ammonium Chloride (TAC), cells were resuspended in flow cytometry wash buffer and stained as normal. For TCR transgenic mice, cells were stained with biotinylated anti-CD4 (Clone GK1.5, homemade) followed by streptavidin-phycoerythrin (SA-PE, Caltag, Burlingame, CA), as well as anti-V_β8.2-fluorescein isothiocyanate (FITC, clone F23.2, homemade). TCR transgenic mice were scored by an increased proportion of $CD4^+$ $V_\beta8.2^+$ lymphocytes in blood compared to non-transgenic littermates. For anti-HEL immunoglobulin transgenic mice, white blood cells were stained with HEL (200 ng/ml in PBS, Sigma) followed by HyHEL9-TriColor (homemade), as well as B220-PE (Clone RA3-6B2, Caltag). Immunoglobulin transgenic mice were scored by an increased proportion of HEL-binding $B220^+$ lymphocytes in blood compared to non-transgenic littermates.

Section 2.4. In vivo cell transfers and immunisations

2.4 (a) Antigen/adjuvant preparation

Intraperitoneal immunisations with HEL (Hen Egg Lysozyme, Sigma) and TEL (Turkey Egg Lysozyme, Sigma) in Complete Freund's Adjuvant (CFA, Sigma) were performed using standard methods (Harlow, 1988). CFA was emulsified in a 1:1 volume ratio with PBS or with PBS containing HEL (or TEL) to give a final protein concentration of 1 mg/ml. The emulsion was formed by vigorous mixing using 3 ml glass syringes connected by a 2-way stopcock. The emulsion was then transferred to 1 ml syringes and injections performed using a 26 gauge needle.

2.4 (b) Immunisation regimes

For immune responses in C57BL/6 recipients, Turkey Egg Lysozyme (TEL) was used, as the C57BL/6 strain is a poor responder to HEL (Hill, 1975) and the transgenic Ig binds TEL with comparable affinity (C. Goodnow, personal communication). Unirradiated C57BL/6 recipient mice were immunised intraperitoneally (i.p.) with 100 μg TEL in CFA. Immunisation was at the time of adoptive cell transfer, or 7 days before in order to prime endogenous recipient T helper cells. Primed mice were boosted with 50 μg TEL in PBS given i.p. or intravenously (i.v.) *via* the lateral tail vein.

In experiments with TCR transgenic helpers, unirradiated (B10.BRxC57BL/6)F_1 recipient mice were immunised i.p. with 100 μg HEL in CFA at the same time as the transfer of TCR transgenic and Ig transgenic cells.

In some experiments unirradiated (B10.BRxC57BL/6)F_1 recipient mice were primed for 6 days before transfer of Ig transgenic cells. In these cases recipients were immunised with 100 μg HEL in CFA at the same time as the transfer of TCR transgenic cells. After 6 days, recipients received Ig transgenic cells and a dose of 100 μg soluble HEL i.v. To stop systemic anaphylactic responses, isoproterenol hydrochloride (Sigma) was given at the same time i.v., at a dose of 4.5 μg/mouse (Han, 1995b).

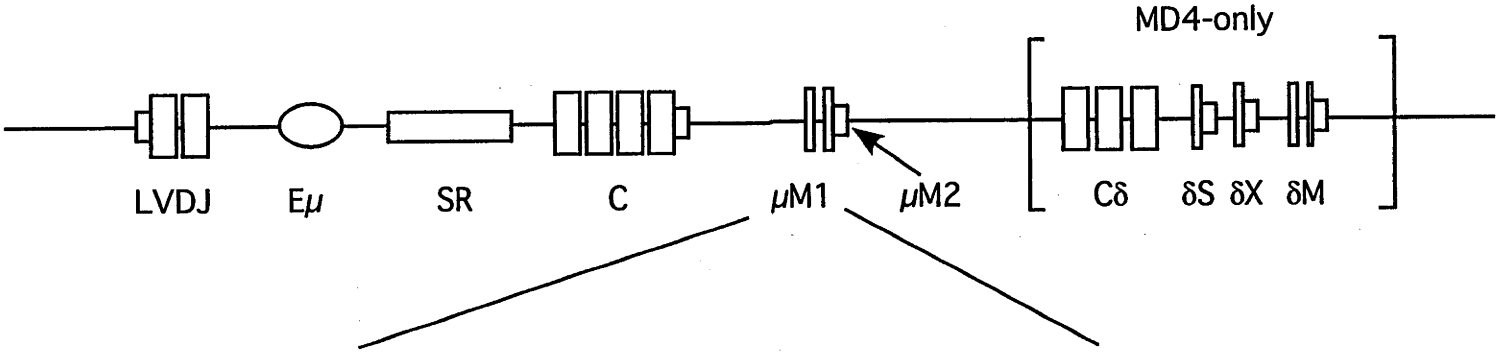
Figure 2-1. Construct information and membrane tail sequence comparisons across the Ig transgenic lines used in this study

The general structure of the anti-HEL immunoglobulin transgenic constructs used to make the panel of mice used in this study is shown. Not drawn to scale. For a representation of the membrane-bound Ig protein, see Figure 1-4.

The sequences of the M1 and M2 exons that encode the extracellular spacer, transmembrane and cytoplasmic sections of the membrane tail are indicated for each transgenic line. Sequence information was taken from previous descriptions of MD4 (Goodnow, 1988; Goodnow, 1989), MM4 (Brink, 1992), MG2, MG6 and MG8 transgenic lines (Pogue, 1996; Pogue, 2000), as well as the constructs used to make the MδE1 and MδE2 lines (Pogue, 1994).

Abbreviations used: **LVDJ**, rearranged heavy chain variable region gene segments from HyHEL10; **E**μ, μ intronic enhancer; **SR** μ, μ switch region; **SR** μ*, μ switch region carrying a 2.8 kb internal deletion; **SR** μ/γ**1**, μ/γ1 hybrid switch region.

Dashes indicate sequence identity with the μ M1 and M2 exon sequences.



Tg line	Hc	SR	Extracellular spacer	Transmembrane region	Cytoplasmic sequence
MD4	μ, δ	μ	EGEVNAEEEGFEN	LWTTASTFIVLFLLSLFYSTTVTLF	KVK (μ Hc)
			GIVNTIQHSCIMDEQSDSYMDL-EENG	--P-MC--VA----T-L--GF--FI	KVK (δ Hc)
MM4	μ	μ	----------	----------	---
MδE1	μ	μ*	EIVNTIQHSCIMDEQSDSYMDL-EENG	----------	---
MδE2	μ	μ*	EIVNTIQHSCIMDEQSDSYMDL-EENG	----------	---
MG2	μ	μ*	EGLQLD-TCAE-QDGELDG	----ITI--S-----VC--AA----	---WIFSSVVELKQTLVPEYKNMIGQAP
MG6	μ	μ*	EGLQLD-TCAE-QDGELDG	----ITI--S-----VC--AA----	---WIFSSVVELKQTLVPEYKNMIGQAP
MG8	μ	μ*	EGLQLD-TCAE-QDGELDG	----ITI--S-----VC--AA----	---WIFSSVVELKQTLVPEYKNMIGQAP
GG4	γ	μ/γ	GLQLD-TCAE-QDGELDG	----ITI--S-----VC--AA----	---WIFSSVVELKQTLVPEYKNMIGQAP

2.4 (c) Transfer of Ig transgenic and TCR transgenic donor cell suspensions into recipient mice

Donor animal spleen suspensions were screened individually by flow cytometry on the day of transfer to confirm PCR screening results and, in conjunction with cell counting, to accurately estimate the number of transgenic cells per spleen. Lysozyme-binding B cells in Ig transgenic donors were enumerated by staining with HEL (200 ng/ml, Sigma), HyHEL9-TriColor and B220-PE (clone RA3-6B2, Caltag). Transgenic T cells in TCR transgenic donors were enumerated by staining with CD4-bio (clone GK1.5) and Vβ8.2-FITC (clone F23.2), followed by SA-PE (Caltag).

Prior to transfer, debris was removed from the donor cell inoculum by layering the cell suspension over a cushion of fetal bovine serum.

Ig transgenic cells alone, or an equal mixture of Ig transgenic and TCR transgenic cells were injected in 200 - 400 µl of RPMI-1640 supplemented with 2 % fetal bovine serum (FBS) (Trace Biosciences, NSW, Australia) and 20 mM HEPES (Sigma) (from this point "complete" RPMI) *via* the lateral tail vein of recipient mice using a 26 gauge needle. Recipient mice were warmed briefly under a heat lamp prior to injection.

Section 2.5. Antibody staining reagents for flow cytometry, ELISA, histochemistry and Western blotting

Name	Clone	Isotype	Conjugation	Source	Step
Avidin			AP	Sigma	2°
B220 (CD45)	RA3-6B2	rt IgG2a	FITC, PE	1, Caltag	1°
CD4	GK1.5	rt IgG2b	bio, APC	1, 1	1°
CD21	7G6	rt IgG2b	bio	1	1°
CD23	B3B4	rt IgG2a	PE	PharMingen	1°
CD138	281-1	rt IgG2a	un	PharMingen	1°
HEL	HyHEL9	m IgG1	FITC, TC, bio	1, 1, 1	1°
rt Ig		rb (pc)	bio	DAKO	2°
Iga (MB-1)		rb (pc)	un	1	1°
Igb (B-29)		rb (pc)	un	1	1°
IgD	11-26	rt IgG2a	un	S.B.	1°
IgD	IgG fraction	shp (pc)	un	T.B.S.	1°
IgDa	AMS9.1	rt IgG2b	FITC	1	1°
IgG	F(ab')2	gt (pc)	HRP	I.C.N.	2°
IgG1		gt (pc)	bio	S.B.	1°

rb IgG		gt (pc)	HRP	Zymed	2°
rt IgG		gt (pc)	HRP	Jackson	2°
shp IgG		rb (pc)	HRP	Jackson	2°
IgM		gt (pc)	AP	S.B.	1°
IgMa	RS3.1	m IgG1	FITC, bio	1,1	1°
kappa Lc		gt (pc)	bio, un	S.B.	1°
Lyn		rb (pc)	un	1	1°
MOMA-1	MOMA-1	rt IgG2a	un	BMA	1°
Streptavidin			AP	Sigma	2°
			CyC	PharMingen	2°
			FITC	Caltag	2°
			HRP	Amersham	2°
			PE	Caltag	2°
p-Tyr	4G10	m IgG2b	un	U.B.	1°
Vb8.2	F23.2	m IgG1	FITC	1	1°

Table 2-1. Antibody staining reagents used in this study.

All target antigens were mouse, unless otherwise indicated.

Abbreviations used: mouse (m); sheep (shp); rat (rt); rabbit (rb); goat (gt); polyclonal (pc); supplied by Prof. C. Goodnow (1); The Binding Site (T. B. S.); Southern Biotechnology Associates Inc. (S. B.); Upstate Biotechnology (U. B.); unconjugated (un); biotinylated (bio); alkaline phosphatase (AP); phycoerythrin (PE); horse-radish peroxidase (HRP);

CyChrome (CyC); TriColor (TC); allophycocyanin (APC); fluorescein isothiocyanate (FITC). "Step" refers to the staining reagent being used as a primary or secondary stain.

Supplier details: Serotec (Oxford, UK), DAKO (Denmark), Jackson ImmunoResearch Laboratories (West Grove, PA), Vector Laboratories (Burlingame, CA), The Binding Site (Birmingham, U.K.), BD PharMingen (San Diego, CA), Southern Biotechnology Associates Inc. (Birmingham, AL), Upstate Biotechnology (Lake Placid, NY), ICN Biomedicals Inc. (Aurora, OH), BMA Biomedicals Inc. (Augst, Switzerland), Zymed Laboratories Inc., San Francisco, CA).

Section 2.6. Cell isolation from mouse tissue

2.6 (a) Cell preparation

Mice were killed by cervical dislocation and spleens or lymph nodes of donor transgenic mice or mice used in phenotyping experiments were removed into 10 ml of complete RPMI. Spleens of recipient mice from adoptive transfer experiments were removed into 2.5 ml of PBS supplemented with 10 % FBS, 1 mM EDTA and 40 μg/ml hyaluronidase (Sigma) in order to fully release activated lymphocytes from the tissue.

In both cases single cell suspensions were prepared by disrupting the spleen capsule between the frosted ends of glass slides. Spleen suspensions from recipient mice were also passed four times through a 26 gauge needle using a 3 ml syringe. The suspensions were made up to 10 ml with complete RPMI, however in some cases, red blood cells were removed by washing once with 10 ml Tris-Ammonium Chloride. Cell suspensions were immediately pelleted by centrifugation at 200 ***g*** for 6 minutes then resuspended in complete RPMI. In all cases spleen suspensions were washed twice in complete RPMI before cell counting.

2.6 (b) Cell counting

Cell counts were performed in two ways, using a hemocytometer for a small number of samples and using a bead-based flow cytometric strategy for large numbers of samples.

For hemocytometer counting, 10 μl of a 1:10 dilution of spleen single cell suspension in white blood cell counting medium (1.5 % acetic acid, 0.01 % gentian violet in ddH_20) was introduced into a hemocytometer (Improved Neubauer type, Hausser Scientific, Horsham, PA). Counting was performed according to standard methods (Harrison, 1997).

For flow cytometry based counting, a 10 µl volume of single cell suspension was mixed with 90 µl flow cytometry buffer containing ~1000 fluorescent beads (Flow-Count, Coulter Corporation, Miami, FL). The sample was passed on a FACScan (BD, Becton Dickinson, San Jose, CA) using a log side scatter setting to visualise the counting beads. Acquisition continued until 200 bead events were counted, then the number of events in a lymphocyte gate set on forward and side scatter was recorded. A single sample was also counted using a hemocytometer. This allowed all samples to be normalised by taking the ratio of lymphocyte events and bead events in each individual sample and multiplying by the absolute counts from the sample counted using a hemocytometer.

Section 2.7. Flow Cytometry

Flow cytometry was performed at the JCSMR FACS facility. For routine analyses, including transgenic mouse blood screening, transgenic donor confirmation, surface phenotyping and flow cytometry-based cell counting, a FacScan (BD) cytometer running CellQuest (BD) software was used to acquire data. For sorting, a FacStar Plus (BD) cytometer was used. For acquisition of 4 colour data, a dual laser FacStar Plus (BD) or LSR (BD) cytometer was used. Spectral overlap compensation was performed in hardware as well as post-acquisition, using WinList v4.0 or v5.0 for Macintosh (Verity Software House, Topsham, ME) (Bagwell, 1993). Subsequent analysis was performed using CellQuest (BD) or FlowJo (Treestar, San Carlos, CA) software.

2.7 (a) Flow cytometry staining

Lymphocytes were stained for flow cytometry in 96-well round bottom plates. After counting, between $5x10^5$ and $5x10^6$ cells were washed once in FACS wash buffer, then resuspended in 50 µl FACS wash containing diluted first step antibodies and incubated at 4 °C for 20 minutes. Samples were made up to 200µl with FACS wash and spun at 670 ***g*** for 6 seconds to pellet the cells. Supernatant was removed by a rapid flick, leaving the cell pellet in a small volume of buffer. After a second wash, cells were resuspended in 50 µl FACS wash containing diluted second step antibodies and incubated at 4 °C for 20 minutes. After washing twice in FACS wash as described above, cell pellets were resuspended in either FACS wash buffer (for immediate analysis) or FACS fixative (for analysis up to 24 hours later) and transferred to polypropylene cluster tubes (Costar Corporations, Cambridge, MA) for acquisition.

2.7 (b) Flow cytometry sorting

Spleen cell suspensions were prepared for cell sorting by isolating donor spleens into complete RPMI with 10 % FBS. Single cell suspensions were prepared as described previously in 2.6 (a). Cells were then washed once into 14 ml of "staining buffer" consisting of 2 % FBS in PBS. Cell suspensions were pelleted and resuspended in 700 µl of first step antibodies diluted in staining buffer, then left at 4 °C for 20 minutes. After staining, the suspension was made up to 14 ml with staining buffer, cells were pelleted and then resuspended in 700 µl (per spleen) second step antibodies diluted in staining buffer. After 20 minutes at 4 °C the cells were washed once in staining buffer then counted, and the cell concentration adjusted with staining buffer to give $5x10^6$ lymphocytes / ml. Cells were filtered through a 60 µm nylon mesh into polypropylene tubes (Costar) prior to sorting.

2.7 (c) Examples of flow cytometry gating strategies

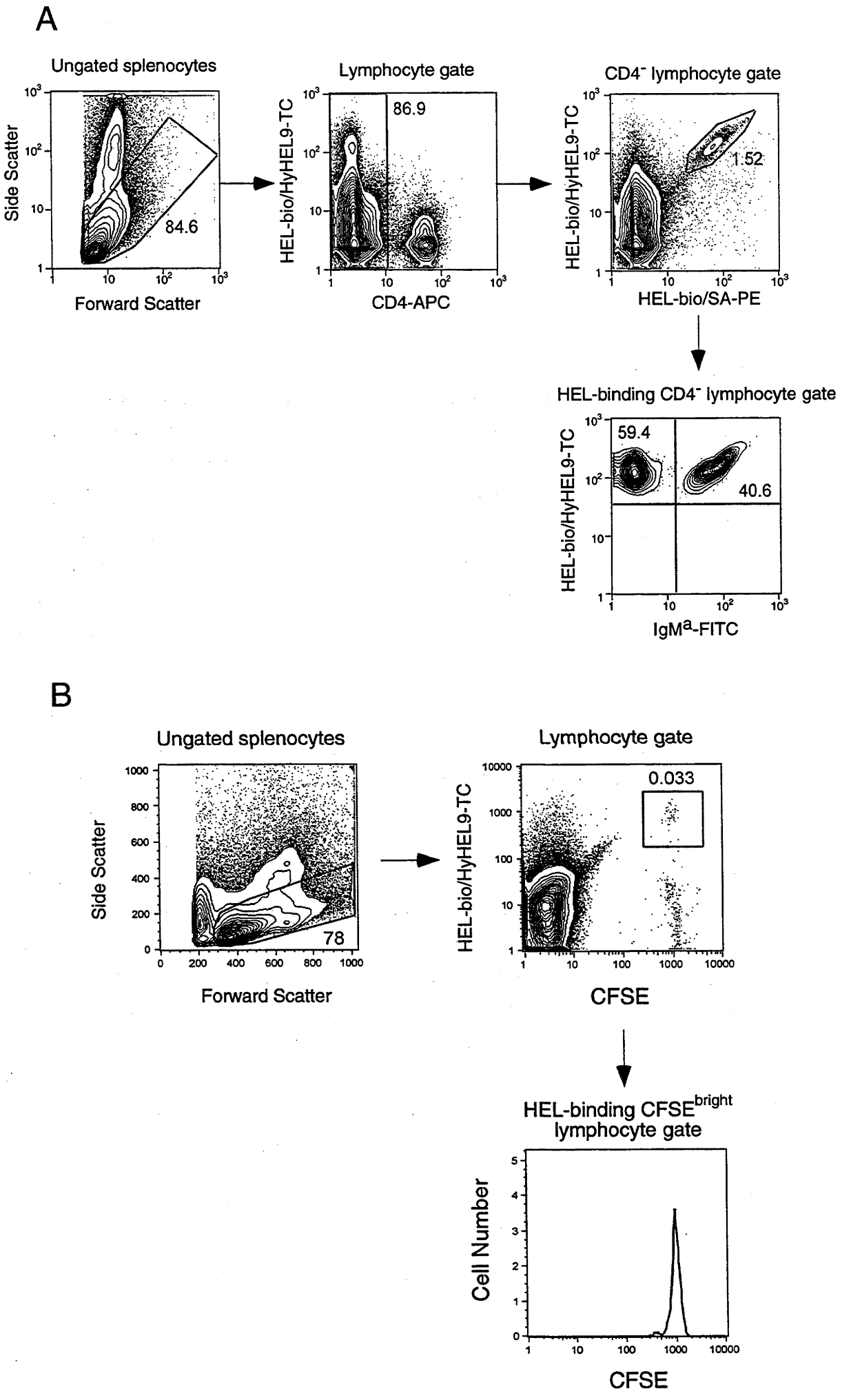
Two gating strategies were used to visualise HEL-binding transgenic B cells amongst the splenic lymphocytes of adoptive transfer recipients, and are shown in Figure 2-2. These varied depending on the frequency of HEL-binding cells present, whether the B cells were CFSE labelled prior to transfer, or whether a co-transfer of two different transgenic B cells was performed. Details of the gating strategies are given in the figure legend.

Figure 2-2. Gating strategies used to visualise HEL-binding B cells in adoptive transfer recipients

Splenocytes were prepared from adoptive transfer recipients as described in Section 2.6. Following the acquisition of flow cytometry files and post-acquisition software compensation, HEL-binding B cells were enumerated in several ways, depending on the experimental design:

A. For recipients that received Ig transgenic B cells, on days 3, 4 and 5 after immunisation, the following set of gates was used. Firstly, a generous lymphocyte gate set on forward and side scatter was used to include lymphoblasts. CD4-APC was then used as a "dump" channel to reduce autofluorescence, so that HEL-binding cells were analysed in the $CD4^-$ lymphocyte gate. HEL-binding cells were then detected by simultaneous staining with HEL-biotin followed by SA-PE and HyHEL9-TC. The HEL-binding cells are present as a tight diagonal population, distinct from autofluorescence (Townsend, 2001). In co-transfer experiments, the HEL-binding $CD4^-$ lymphocytes were queried for IgM^a expression as shown. This was used to distinguish IgM^{a-} IgG (GG4) B cells from co-transferred IgM (MM4) or IgM/G (MG2) B cells, both of which express IgM^a. If the transgenic B cells were CFSE-labelled prior to transfer, they could also be queried for their CFSE dilution profile using the HEL^+ $CD4^-$ lymphocyte gate.

B. For recipients that received CFSE-labelled transgenic B cells, a different gating strategy was employed to detect the cells on days 1 and 2 after immunisation and transfer, while HEL-binding cells were still rare. It was found that detecting CFSE-labelled HEL-binding B cells on the basis of CFSE and HEL/HyHEL9-TC staining was most sensitive, resolving the transferred $CFSE^{bright}$ cells as a distinct population. In this example, CFSE-labelled IgM/G (MG2) transgenic B cells on day 1 after transfer into immunised recipients were detected by first gating on lymphoblasts amongst total splenocytes. Then, $CFSE^{bright}$ HEL-binding lymphocytes were gated. The level of CFSE expression in the transferred transgenic B cells was then expressed as a histogram. In some cases the $CFSE^{bright}$ HEL-binding gate was set on the $CD4^-$ lymphocyte population as described in (A).


A
Ungated splenocytes
Side Scatter
Forward Scatter
84.6
Lymphocyte gate
HEL-bio/HyHEL9-TC
CD4-APC
86.9
CD4⁻ lymphocyte gate
HEL-bio/HyHEL9-TC
HEL-bio/SA-PE
1.52
HEL-binding CD4⁻ lymphocyte gate
HEL-bio/HyHEL9-TC
IgM^a-FITC
59.4
40.6
B
Ungated splenocytes
Side Scatter
Forward Scatter
78
Lymphocyte gate
HEL-bio/HyHEL9-TC
CFSE
0.033
HEL-binding CFSE^bright lymphocyte gate
Cell Number
CFSE

Section 2.8. CFSE Labelling

Labelling of donor splenocytes with 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) was performed as described elsewhere (Lyons, 1994; Warren, 1999), with minor modifications. Single cell suspensions were stained at room temperature for 7 minutes in a final CFSE concentration of 2.5 μM or 10 μM in complete RPMI-1640 (10 % FBS). The labelling reaction was halted by addition of > 2 volumes of ice-cold complete RPMI-1640 (10 % FBS). Cells were washed extensively in complete RPMI-1640 (10 % FBS) at 4 °C then resuspended in complete RPMI-1640 (2 % FBS) prior to adoptive transfer. In recipients that received CFSE-labelled transgenic B cells, donor B cells were tracked using CFSE and HEL / HyHEL9-TriColor on day 1 and day 2 of the immune response as described in Figure 2-2, part B. On days 3, 4 and 5, transgenic B cell net clonal expansion was determined by staining with two fluorochromes for HEL simultaneously (Townsend, 2001) as described in Figure 2-2, part A.

Section 2.9. ELISPOT

ELISPOT assays were performed as described (Sedgwick, 1986) using biotinylated goat anti-mouse IgG_1, biotinylated anti-IgM^a (Clone RS3.1) and avidin-alkaline phosphatase (Sigma).

Briefly, 1 mg/ml HEL in 0.05 M bicarbonate buffer (pH 9.6) was bound to 24-well tissue culture plates (Linbro, ICN Biochemicals, Aurora, OH). After blocking with 1 % BSA (Fraction V, Sigma) in PBS, spleen or bone marrow single cell suspensions were added and cultured for four hours at 37 °C in a 5 % CO_2 incubator. After washing with PBS and PBS supplemented with 0.05 % Tween-20, the plates were blocked with a mixture of BSA and skim milk powder at 37 °C for 30 minutes. First step biotinylated antibody was added at 4 °C overnight, followed by second step avidin-alkaline phosphatase at 37 °C for 1 hour. Plates were developed using 5-bromo-4-chloro-3-indoyl phosphate (BCIP, Sigma). One volume of 3 % (w/v) agarose was mixed with 4 volumes of 1.25 mg/ml BCIP in 2-amino-2-methyl-1-propanol buffer (AMP, Sigma), then the mixture added to the plates. After colour development, dilutions showing ~200 spots were counted manually using a dissecting microscope.

Section 2.10. Serum ELISA

Anti-lysozyme antibodies in sera were measured as previously described (Rathmell, 1994), in this case using alkaline phosphatase conjugated goat anti-mouse IgM, biotinylated goat anti-mouse IgG_1 or biotinylated goat anti-mouse kappa (all from Southern Biotechnology), followed by avidin-alkaline phosphatase (Sigma).

Briefly, 96-well flat bottom plates (Maxisorb, Nunc A/S, Roskilde, Denmark) were coated with HEL (10 µg/ml) in 0.05 M bicarbonate buffer (pH 9.6) overnight. Plates were blocked with 0.1 % BSA in PBS for 2 hours at 37 °C. Serum dilutions prepared in 50 µl of 0.1 % BSA/PBS were applied to the plate, covered with adhesive plastic and incubated at 37 °C for 2 hours. First step antibodies were diluted in 0.1 % BSA in PBS, and 50 µl added per well. Plates were incubated at 37 °C for 1-2 hours. Secondary antibodies were diluted in 0.1 % BSA in PBS and 50 µl added per well. Plates were incubated at 37 °C for 1 hour. To develop, nitrophenyl phosphate (5 mg tablets, Sigma) was prepared at 1 mg/ml in NPP buffer and 100 µl added to each well. After visible colour change, absorbance was measured at 405 nm and 650 nm using a 96-well plate reader (THERMOmax, Molecular Devices Corp., CA). Plates were washed extensively with 0.5 % Tween-20 in PBS in between each step.

Reference sera from IgM and IgG transgenic mice containing IgM or IgG_1 anti-lysozyme antibodies with the same V regions and kappa chains were used to standardise the ELISA. The same pool of reference sera was used throughout all these studies and was frozen in small aliquots at -80 °C then each aliquot thawed once. The IgG transgenic reference serum was assigned a value of 10,000 anti-lysozyme kappa units and the corresponding level of anti-lysozyme kappa units in the IgM transgenic reference sera was determined in parallel using the same anti-mouse kappa detecting reagent. IgG and IgM standard curves were constructed using these reference sera detected with anti-mouse IgM and anti-mouse IgG reagents on each plate used to measure the test sera at multiple dilutions. Lysozyme-binding IgM and IgG levels in any test serum could then be related back to the IgG and IgM standard curves, for which the level of anti-lysozyme kappa units was known.

Section 2.11. Histology

Spleens were removed and half of the tissue taken for histology. This was immediately snap frozen in liquid nitrogen for sectioning, then stored in tin foil at -80 °C. For sectioning, spleens were mounted on a cold metal chuck using OCT compound (Sakura

Finetek, Torrance, CA) and placed in a cryostat (Bright Instrument Co. Ltd., Huntingdon, U.K.) chilled to - 20 °C. After carefully exposing the spleen using a scalpel, 5-7 μm sections were cut and mounted on glass slides. Sections were allowed to air dry and were then fixed in ice cold acetone at 4 °C for 20-30 minutes. After acetone fixing, sections were sealed in small plastic bags and stored at -20 °C until staining.

Sections were rehydrated in Tris-buffered saline (TBS) for 10 minutes before staining. Staining was performed at room temperature in a humidified chamber and incubation times were 1 hour for all staining steps except for the 1 ° and 2 ° stains for HEL-binding cells that were 30 minutes each. All staining reagents were diluted in TBS with 0.1 % BSA. All sections were washed twice in TBS between staining steps. All secondary reagents were incubated with 10 % normal mouse serum in TBS for 30 minutes to decrease non-specific staining of mouse tissue. The sequence of stains for each target antigen is given in

Table 2-2.

Target	1 °	2 °	3 °	4 °	5 °	DAB	Fast Blue
HEL-binding cells	HEL	HyHEL9-bio	HEL	HyHEL9-bio	SA-AP	-	Yes
CD138 (Syndecan)	rt-α-mCD138	rb-α-rt bio	SA-AP	-	-	-	Yes
IgD (1)	rt-α-mIgD	gt-α-rt HRP	-	-	-	Yes	-
IgD(2)	shp-α-mIgD	rb-α-shp HRP	-	-	-	Yes	-
MOMA-1	rt-α-mMOMA-1	gt-α-rt HRP	-	-	-	Yes	-

Table 2-2. Schematic showing the staining and developing steps for immunohistochemical detection of various target antigens.

Abbreviations: **rt** rat, **m** mouse, **shp** sheep, **rb** rabbit, **gt** goat, **HRP** horse radish peroxidase, **PNA** peanut agglutinin, **SA-AP** streptavidin-alkaline phosphatase.

Sections were developed sequentially. Firstly, peroxidase-labelled antigens were developed using diaminobenzidine (DAB, Pierce, Rockford, IL) solution at 1 mg/ml in TBS with a 1:1000 dilution of hydrogen peroxide (from a 30 % solution, Pierce). DAB solution was filtered through a 0.45 μm syringe mounted filter (Millipore, Bedford, MA) directly onto the section. Secondly, alkaline-phosphatase labelled antigens were developed using a Fast Blue kit (Vector), following the manufacturers instructions. Levamisole (Sigma) was added at 1 μM to the Fast Blue reagent to inhibit endogenous alkaline phosphatase activity.

After washing with ddH_20 sections were air-dried and mounted using Immumount (Shandon Inc., Pittsburgh, PA).

Section 2.12. Biochemistry techniques

2.12 (a) Cell stimulation and lysis

After counting and TAC lysis, cells were resuspended in complete RPMI-1640 at a concentration of 10^7 per 200 μl. Cells were allowed to warm at 37 °C for 3 minutes prior to stimulation. Cells were then stimulated ± HEL at 10 μg/ml for 3 minutes. Different lysis conditions were used from this point, depending on the desired cell fractionation. In all cases the 1x lysis buffer contained the following inhibitor cocktail: protease inhibitors - 2.5 mM PMSF (Sigma), 200 μM leupeptin hemisulfate (Calbiochem-Norabiochem Corp., La Jolla, CA), 200 μM aprotinin (Sigma) and phosphatase inhibitors - 10 μM sodium orthovanadate (Sigma, pre-activated using H_2O_2), 100 μM phenylarsene oxide (Sigma), 25 mM NaF (Sigma)

Cytoskeletal stabilising buffer (CSB) lysis

After stimulation, cells were pelleted by spinning at 850 ***g*** for 3 minutes in a benchtop microfuge. Media was aspirated and the pellet washed once in 200 μl complete RPMI-1640. After washing, 200 μl "low salt" CSB buffer (+ inhibitors) was added, the pellet resuspended and the lysate left at 4 °C for 10 minutes. The "low salt" soluble cell fraction was recovered as supernatant after spinning for 10 minutes (380 ***g***, 4 °C) and immediately added to 50 μl of boiling 3x sample buffer (1x sample buffer contained: 2 % (w/v) SDS, 10 % (v/v) glycerol, 62.5 mM Tris.HCl (6.8), 0.01 % (w/v) bromophenol blue, ± 3.3 % (v/v)

2-mercaptoethanol). The sample was boiled for 5 minutes then snap frozen in dry ice/MeOH and stored at –70 °C. CSB buffer components are described in Section 2.13.

After washing once in 100 µl "low salt" CSB buffer, the detergent-insoluble pellet was resuspended in 100 µl "high salt" CSB buffer and the lysate left at 4 °C for 10 minutes. The "high salt"-extractable material was recovered from the detergent-insoluble pellet after centrifugation for 10 minutes (380 ***g***, 4 °C) and immediately added to 25 µl of boiling 3x sample buffer. The sample was boiled and stored as above.

The lysate pellet, corresponding to detergent insoluble cytoplasmic and nuclear fractions was added to 100 µl of boiling 1x sample buffer. The sample was boiled and stored as above.

TX-100 lysis

After HEL stimulation, cells were pelleted by centrifugation at 850 ***g*** for 3 minutes in a benchtop microfuge. After aspirating the supernatant, the pellet was resuspended in 200 µl TX-100 lysis buffer and left on ice for 10 minutes. TX-100 lysis buffer comprised 1% (v/v) TX-100, 10 mM EDTA, 50 mM Tris.HCl (pH 7.5), 250 mM NaCl, as well as the inhibitor mixture described above.

After lysis, samples were spun at 16000 ***g***, 4 °C, in a benchtop centrifuge (Labofuge 400R, Heraeus Instruments, New Jersey). The 200 µl supernatant fraction corresponding to detergent-soluble cell structures was immediately added to 100 µl boiling 3x sample buffer for 5 minutes. Also, the detergent insoluble pellet was solubilised by boiling for 5 minutes in 100 µl 1x sample buffer. Both fractions were snap frozen in dry ice/MeOH and stored at –70 °C until analysis.

2.12 (b) Western Blotting

Discontinuous polyacrylamide gel electrophoresis under reducing or non-reducing conditions was used to separate proteins within detergent lysates. Gels were prepared using standard recipes (Sambrook, 1989) in the Mini-Protean II mini-gel format (BioRad). Samples were thawed, boiled for 5 minutes and spun at 16000 ***g*** in a benchtop microfuge to clarify insoluble material prior to loading. Coloured broad range molecular mass standards (BioRad) were run in parallel to estimate sample protein molecular mass. These ranged from 20-200 kDa apparent molecular mass when run on a 10 % polyacrylamide gel. These were also boiled and centrifuged prior to loading.

Western blotting to a PVDF membrane (Immobilin P, 0.45 μm, Millipore, Bedford, MA) was performed according to standard methods, using the Mini-Protean II (BioRad) mini-gel format. Blotting was performed overnight at 40 Volts or over 1 hour at 100 Volts, in both cases at 4 °C.

After blotting, the membrane was blocked overnight or for 2 hours at room temperature with 3 % (w/v) BSA dissolved in TBS/Tween (TBS for Western blot – 20 mM Tris.HCl (pH 7.5), 100 mM NaCl, 2 mM EDTA, with 0.1 % (v/v) Tween-20). The blocking solution was syringe-filtered (0.45 μm, Millipore) prior to use. After 3x5 minute washes in TBS/Tween, primary antibody diluted in TBS/Tween with 1 % (w/v) BSA and 0.1 % sodium azide was added to the blot and incubated for 1 hour at room temperature. The primary antibody was saved for reuse, and after washing the HRP-conjugated secondary antibody was diluted in TBS/Tween and added for 30 minutes at room temperature. After the final wash, blots were developed using chemiluminescence. The chemiluminescent substrate (Rennaisance, NEN, Boston, MA) was prepared and incubated for 30 seconds with the blot. After removing the excess substrate, the blot was wrapped in cling-film, exposed to X-ray film in a cassette (X-OMAT AR, Eastman Kodak Co., Rochester, NY), and developed.

Section 2.13. Buffers, media and stock solutions

Buffers

(1) AMP Buffer

1 M	2-amino-2-methyl-1-propanol (Sigma)
0.7 mM	$MgCl_2$
0.01 % (v/v)	TX-100
0.1 % (w/v)	Sodium azide
pH 10.25	

(2) Carbonate buffer

$NaHCO_3$ 1.59 g/L, Na_2CO_3 2.93 g/L dissolved in ddH_20, pH 9.6

(3) Tris Buffered Saline (TBS) for histology

60.6 g Trizma.HCl (Sigma)

13.9 g Trizma Base (?)

87.7 g NaCl

Dissolved in 10 L ddH_2O, pH 7.6

(4) Phosphate Buffered Saline (PBS) – prepared in house, JCSMR media

8.0 g/L NaCl

1.25 g/L Disodium hydrogen orthophosphate

0.35 g/L Sodium dihydrogen orthophosphate (monohydrate)

(5) FACS wash solution

3 % (v/v) FBS and 0.1 % (w/v) sodium azide in PBS. Stored at 4 °C.

(6) FACS fixative

1 % (w/v) paraformaldehyde in PBS. Stored at 4 °C.

Media

(1) RPMI 1640 – prepared in house, JCSMR media

10.44 g/L RPMI powder (GibcoBRL, Life Technologies, NY, USA)

2.0 g/L Sodium bicarbonate

Solution microfiltered (0.2 μm) and aseptically dispensed into sterile Schott bottles.
pH 7.3-7.5.

Stock solutions

(1) Alsever's solution (modified) – prepared in house, JCSMR media

For a 1 L stock:

8 g	sodium citrate
4.2 g	sodium chloride
20.5 g	glucose
0.8 g	citric acid

Dissolve ingredients in 1 L ddH_20. Adjust pH to 6.1 with 10 % citric acid. Autoclave and store at 4 °C.

(2) Nitrophenyl phosphate (NPP) buffer

15 mM	Na_2CO_3
35 mM	$NaHCO_3$
0.1 mM	$MgCl_2$
0.1 % (w/v)	sodium azide

pH 9.8

(3) Tris Ammonium Chloride (TAC) – Red blood cell lysis buffer

Lysis buffer contains 0.02 mM Tris and 0.1 M ammonium chloride in PBS.

1.03 g Tris and 3.375 g ammonium chloride dissolved in 500 mL PBS.

pH adjusted to 7.2 with conc. HCl.

Autoclaved and stored at room temperature.

(4) Cytoskeletal Stabilising Buffer (CSB)

0.1 % (v/v) TX-100 (Sigma)

20 mM HEPES (Sigma)

300 mM Sucrose

0.1 mg/ml BSA

20 mM sodium chloride ("low salt" buffer) **OR** 150 mM sodium chloride ("high salt" buffer)

5 mM magnesium chloride

prepared in ddH_20

Chapter 3. The IgG membrane tail is a regulator of B cell clonal expansion *in vivo*

Section 3.1. Introduction

Memory antibody responses to T-dependent antigens are characterised by the rapid production of high titres of antigen-specific antibody (Burnet, 1959). The prevailing view is that antigen-specific memory B cells are maintained after clonal expansion during the primary immune response and are responsible for the features of the secondary response (Celada, 1971; Gray, 1993; Sprent, 1994; Ahmed, 1996).

Defining the basis for these heightened memory responses has been complicated, however, because the first priming with antigen brings about numerous interconnected changes in the antigen-specific B cell pool. For example, clonal expansion increases the frequency of antigen-specific B cells (Hayakawa, 1987; Schittek, 1990) and antigen-driven selection in germinal centres leads to the appearance of B cells bearing somatically mutated high affinity antigen receptors (Eisen, 1964; Weiss, 1990). Also, phenotypic changes such as the altered expression of cell surface molecules (Klein, 1998; Tangye, 1998), modified homing patterns (Liu, 1991; Liu, 1995; Tierens, 1999) and lower activation requirements (Liu, 1995) are characteristic of memory B cells. In addition, isotype switching during the formation of many memory B cells leads to the replacement of surface IgM and IgD with downstream isotypes, notably IgG in the case of protein antigens.

Isotype switching to IgG is an irreversible molecular change that has been used in many studies as a marker for memory B cells (Hayakawa, 1987; Maruyama, 2000). The expression of switched isotypes confers a significant alteration in the membrane Ig B cell antigen receptor (BCR), as each carry a distinct extracellular spacer, transmembrane and extended cytoplasmic tail sequence that are highly conserved within isotypes and between species (Reth, 1992; Nussenzweig, 1997) (Figure 1-3). Relatively few studies have investigated the signalling consequences of isotype switching, however, especially with respect to the function of the conserved membrane tail during memory B cell responses *in vivo*.

In vitro studies of the function of switched isotype receptors have focussed on a conserved YXXM motif in the cytoplasmic tail sequence of IgG and IgE and its role as a putative internalisation signal for antigen processing and presentation. It has been reported that both IgG_{2a} (Weiser, 1994) and IgG_1(Knight, 1997) isotype antigen receptors can be internalised when expressed in cell lines that lack the accessory molecules Igα and Igβ and that antigen presentation *in vitro* by IgG_1 isotype receptors expressed without Ig$\alpha\beta$ is partially disrupted by mutation of the YXXM motif (Knight, 1997). The physiological significance of these findings is not clear however, as mutation of the YXXM motif has no

effect on the rate of antigen presentation *in vitro* when IgG_1 is associated with Igαβ (Knight, 1997). Also there is no indication *in vivo* that IgG isotypes are expressed at any stage of B cell development in the absence of Igαβ.

Recent studies using gene-targeted mice which lack either IgG_1 or IgE cytoplasmic tail sequences have established that these segments are needed by B cells expressing these isotypes *in vivo*, although the role they serve has not been identified. Mice carrying a targeted disruption of the cytoplasmic tail sequences of IgG_1 (Kaisho, 1997) or IgE (Achatz, 1997) generated poor antigen-specific IgG_1 or IgE primary and secondary antibody responses. For IgG tail-truncation mutant mice, profound differences in antigen receptor affinity and responding IgG cell frequency existed between mutants and wild type mice. This raises the possibility that cells with truncated receptors had either poor activation, increased cell death, a defect in germinal centre formation or a lower rate of cell division. Kaisho *et al.* (Kaisho, 1997) found lower surface expression of the truncated Δtail IgG BCR on unselected lipopolysaccharide (LPS) blasts *in vitro*, suggesting the alternative explanation that the IgG tail is needed for adequate surface expression of this isotype rather than for a distinct function of IgG BCRs.

To investigate how BCR isotype switching affects the way B cells respond to antigen *in vivo*, independent of changes in specificity, frequency and priming, we have examined the response of B cells from immunoglobulin gene (Ig) transgenic mice. We have used a panel of transgenic mice which all share the same high affinity variable region specific for the protein antigen Hen Egg Lysozyme (Goodnow, 1988; Brink, 1992; Pogue, 2000) (HEL). The BCR isotypes expressed are IgM or IgD, which are characteristic of naïve B cells, or are IgG, an isotype strongly associated with B cell memory. In addition to B cells expressing native IgM or IgG BCRs, we also engineered transgenic strains with HEL-binding B cells expressing a chimeric IgM/G BCR, where the exons of the secreted product are from IgM but the membrane tail exons are from IgG (Pogue, 2000) (see Figure 3-1 part A and Figure 2-1).

This strategy avoids the problems interpreting loss of function results from truncated receptors, because we test whether any unique functions of the IgG membrane tail can be gained by IgM through the transfer of these segments. By seeding transgenic B cells from these lines into normal immune responses, it becomes possible to control for many variables. Firstly, precursor frequency can be precisely controlled. Also, the responding B cells are all naïve and can be prepared at matched stages of development, bearing antigen receptors of a defined isotype with an identical affinity for antigen. Finally, the use of high affinity transgenic B cells allows clonal expansion and differentiation to be followed at the single cell level by flow cytometry.

Using this strategy, it was found that changing the BCR isotype from IgM to IgG confers a greatly increased production of plasma cells during extrafollicular T cell-dependent antibody reactions. The IgG membrane tail is sufficient to confer this burst-enhancing property on IgM, and the enhanced response results from decreased attrition of progeny cells rather than enhanced activation or proliferation.

Section 3.2. IgG-bearing B cells produce more antibody than IgM-bearing B cells during an immune response

To test the effect of high-affinity precursor frequency and antigen receptor isotype on antibody production during a primary immune response, varying numbers of naive lysozyme-binding B cells from IgM isotype and IgG isotype donors were seeded in a 1:1 ratio into unirradiated C57BL/6 recipient mice immunised with lysozyme. This co-transfer design had the advantage of controlling for any other differences affecting helper T cells or other cells, although the same results were obtained seeding IgM and IgG cells into separate recipients.

Serum was collected from the primary response on day 10 after immunisation and from the secondary response 5 days after boosting. The magnitude of the anti-lysozyme IgM and IgG immune response was then determined by ELISA (Figure 3-1, part B). The kappa light chains shared in common by the transgenic IgM and IgG were used to standardise the assay to allow the amounts of each to be directly compared (see Section 2.10, materials and methods). The endogenous primary and secondary IgM responses were of low titre and were not increased by transfer of up to 10^6 transgenic B cells bearing the high-affinity antigen-specific IgM isotype BCR. By contrast, as few as 10^4 transgenic B cells bearing high-affinity antigen-specific receptors of the IgG isotype increased the primary IgG response ~400 fold. Groups of mice that received 10^5 or 10^6 IgG B cells made a primary anti-lysozyme IgG immune response that equalled the magnitude of the endogenous secondary response to lysozyme.

Section 3.3. The IgG membrane tail is the molecular determinant responsible for heightened antibody responses

The role of the unique membrane tail of IgG in this heightened antibody response was tested by comparing the response of IgG B cells to B cells bearing IgM or the chimeric IgM/G receptor. After seeding into a primary antibody response, the IgM/G cells mounted

a comparable serum antibody response to the IgG cells that was 40-fold higher than the IgM cells (Figure 3-1, part C).

In additional experiments, C57BL/6 recipient mice were primed with lysozyme to expand and differentiate the pool of helper T cells, then mixtures of IgG and IgM, or IgG and IgM/G transgenic B cells were co-transferred and the recipients were boosted. Antibody production was assessed by ELISPOT assay to enumerate splenic antibody-forming cells (AFCs). It was found that IgM transgenic B cells made 10-fold fewer AFCs than IgG transgenic B cells, whereas IgM/G cells made comparable numbers of AFCs to IgG cells (Figure 3-2, part A). Data pooled from a number of experiments confirms that IgM transgenic B cells made 10-fold fewer AFCs compared to IgG or IgM/G transgenic B cells in this system (Figure 3-2, part B). These data identify the IgG membrane tail region as the crucial domain that leads to the large differences in serum antibody production and splenic AFC numbers between IgG and IgM transgenic cells during an immune response.

Figure 3-1. Seeding a primary antibody response with naïve B confers memory-response characteristics when the receptors contain the unique IgG membrane tail

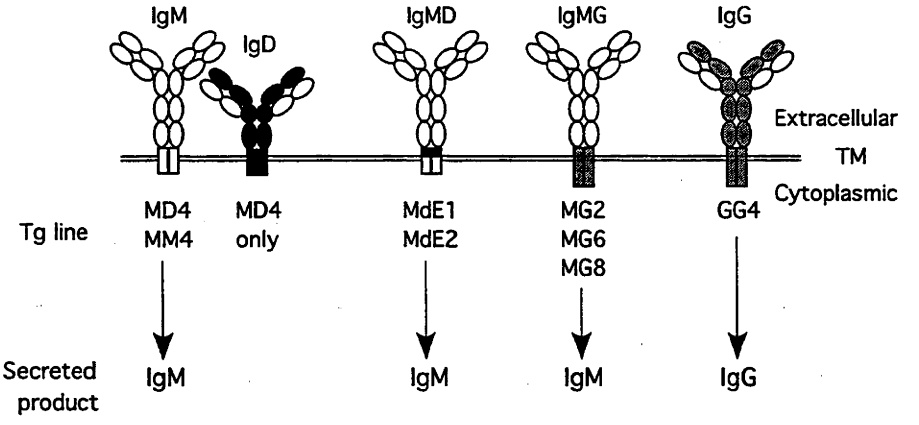
A. Schematic illustrating membrane Ig sequence differences in the carboxy-terminal membrane tails of mouse IgM, IgD and IgG_1. Diagrams below show the transgenic lines used in this chapter, transgenic BCR structures and secreted immunoglobulin isotypes.

B. Mixtures of splenocytes containing equal numbers of IgG and IgM HEL-binding transgenic B cells were prepared. Inocula containing 10^6, 10^5, 10^4, or 10^3 of each type of HEL-binding B cell was injected into recipient C57BL/6 mice that were immunised with lysozyme in CFA (i.p.) and boosted with soluble lysozyme (i.v.) 21 days later. Filled circles show serum anti-lysozyme IgG_1 levels and open circles give serum anti-lysozyme IgM levels in individual animals on day 10 and day 26 as determined by ELISA (see Section 2.10 for a description of anti-lysozyme kappa units). The limit of detection for anti-lysozyme IgM was 100 kappa units and the limit of detection for anti-lysozyme IgG was 2 kappa units.

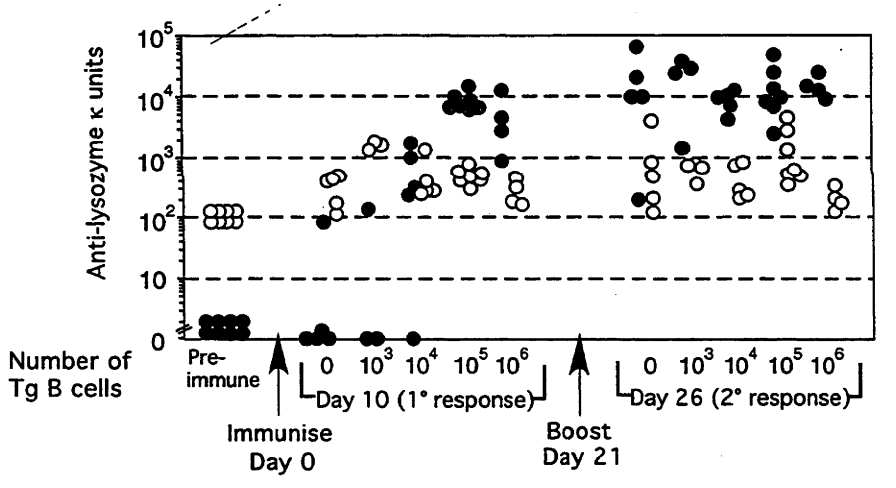
C. As in (B), except 10^6 lysozyme-binding B cell mixtures of the indicated types were transferred and the serum response measured on day 10 by ELISA.

A

	Extracellular spacer	Transmembrane sequence	Cytoplasmic sequence
Mouse IgM	EGEVNAEEEGFEN	LWTTASTFIVLFLLSLFYSTTVTLF	KVK
Mouse IgD	GIVNTIQHSCIMDEQSDSYMDL-EENG	--P-MC--VA----T-L--GF--FI	KVK
Mouse IgG_1	EGLQLD-TCAE-QDG-ELDG	----ITI--S-----VC--AA----	---WIFSSVVELKQTLVPEYKNMIGQAP



B



C

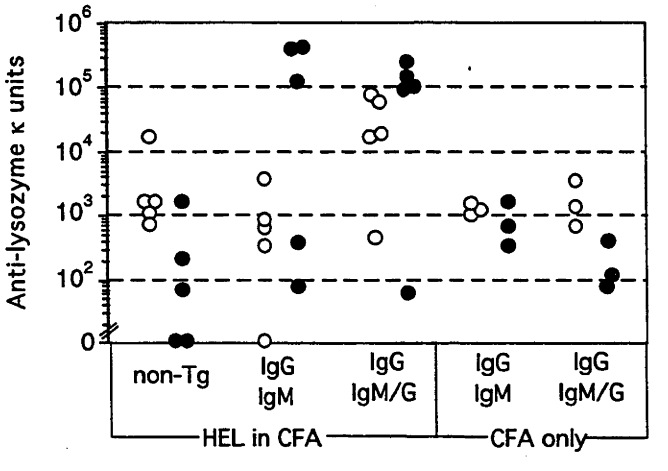
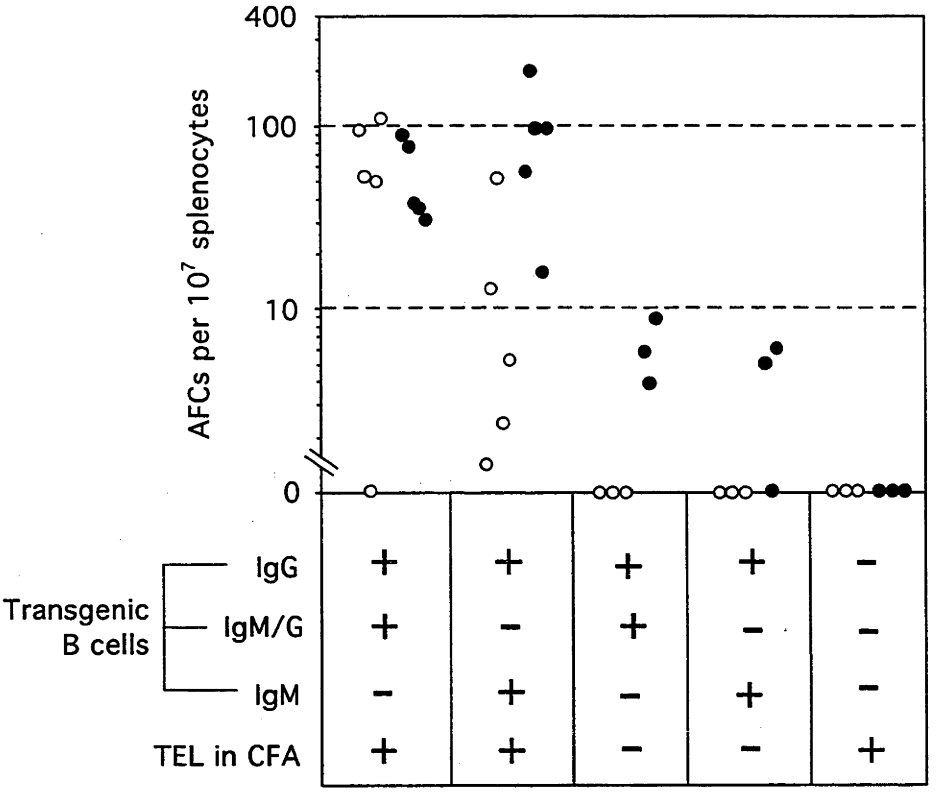


Figure 3-2. The IgG membrane tail increases the production of antibody-forming cells

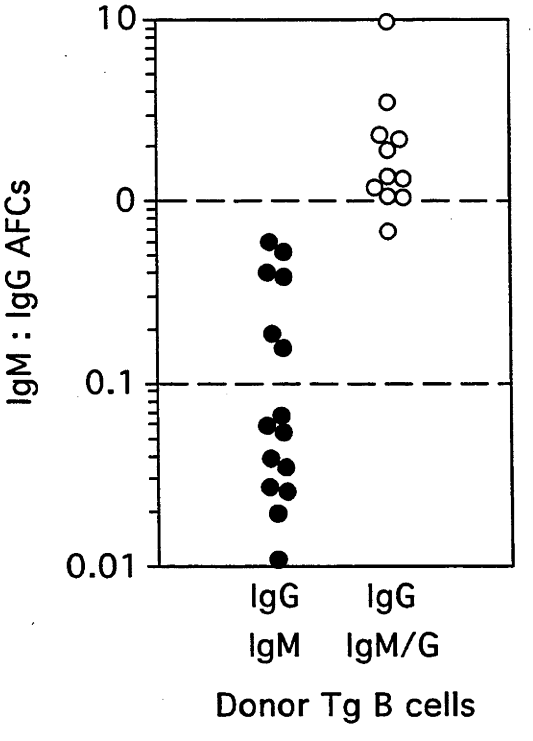
A. C57BL/6 recipient mice were immunised with TEL in CFA to prime helper T cells. Seven days later each recipient received mixtures of 10^6 IgG transgenic B cells with either 10^6 IgM or 10^6 IgM/G transgenic B cells and was boosted with soluble TEL in PBS (i.v.). Recipient splenocytes were isolated 5 days after transfer and lysozyme-binding AFCs enumerated by ELISPOT. Filled circles give the anti-lysozyme IgG_1 AFC levels and open circles give the anti-lysozyme IgM^a AFC levels per 10^7 recipient lymphocytes.

B. Ratio of lysozyme-specific IgM^a AFCs to lysozyme-specific IgG_1 AFCs from individual mice. Filled circles show the ratio of IgM:IgG AFCs in recipients that received a mixture of IgG and IgM transgenic B cells. Open circles show the ratio of IgM:IgG AFCs in recipients that received a mixture of IgG and IgM/G transgenic B cells. Data was pooled from several transfer experiments performed as described in (A).

A


400
100
10
0
AFCs per 10^7 splenocytes
Transgenic B cells
IgG
IgM/G
IgM
TEL in CFA
+ + + + -
+ - + - -
- + - + -
+ + - - +

B


10
0
0.1
0.01
IgM : IgG AFCs
IgG IgM
IgG IgM/G
Donor Tg B cells

Section 3.4. The IgG tail augments B cell clonal expansion during an immune response

Heightened antibody production from IgG or IgM/G cells could have been due to either the greater clonal expansion of those cells or the differentiation of a greater fraction into AFCs. These ideas were tested by seeding the primary antibody response with large numbers of lysozyme-specific helper T cells from 3A9 TCR transgenic mice (Ho, 1994), as well as lysozyme-specific B cells. Provision of greater T cell help allowed a large fraction of the transferred B cells to be activated synchronously after immunisation, making it possible to compare directly the initial proliferation of the different B cells. As before, IgG or IgM/G B cells produced 100-fold more AFCs than IgM B cells (Figure 3-3, part A). MD-4 transgenic B cells that co-express IgM and IgD naive isotypes (Goodnow, 1988; Goodnow, 1989) also yielded relatively few AFCs, at least 10-fold fewer than the IgG or IgM/G B cells. Flow cytometric enumeration of the HEL-binding B cells (Figure 3-3, parts B and C) showed that the difference in AFC production is mostly explained by an increased net clonal expansion of IgG or IgM/G B cells compared to the IgM or IgM/IgD isotype B cells.

Importantly, comparisons have been made using four lines expressing the IgG membrane tail (GG4, MG2, MG6 and MG8, Pogue, 2000), as well as four IgM or IgD transgenic lines which express either IgM or IgD alone (MM4, Brink, 1992), co-express IgM and IgD (MD4, Goodnow, 1988) or express IgM with the 27-amino acid extracellular spacer domain of IgD (MδE1 and MδE2, Pogue, 1994). These lines were made with constructs that differed in the type and the extent of the transgene switch regions. In every case, the transgenic lines expressing the IgG membrane tail make at least a 10-fold greater immune response *in vivo* on the basis of clonal expansion and AFC production (Figure 3-4). This strongly supports the conclusion that expression of the IgG membrane tail confers enhanced reactivity to antigen *in vivo*.

The possibility that line to line variation in transgenic constructs as well as the effect of IgD expression in this system will be considered in more detail in Chapter 6.

Section 3.5. Increased clonal expansion conferred by the IgG membrane tail is due to decreased death over successive cell divisions

The much larger clonal expansion conferred by the IgG membrane tail region could have reflected a greater fraction of cells activated initially by helper cells, a greater rate of cell division leading to many more progeny, or a lower attrition of progeny cells. To distinguish between these possibilities transgenic B cells were loaded with the cell-division tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Lyons, 1994) prior to transfer. Cell division was then followed by flow cytometry on day 1, day 2 and day 5 after immunisation (Figure 3-5). There was no evidence for division of the transferred transgenic B cells on day 1. On day 2, a comparable fraction of the IgM, IgG and IgM/G B cells had divided, shown by dilution of CFSE on a fraction of cells relative to controls. This suggests there is no difference in the initial activation of the IgM, IgG and IgM/G B cells by the available T cell help. By day 5 however, the net clonal expansion of the IgG and IgM/G cells was much greater than the IgM cells. On day 5 the remaining IgM B cells had nevertheless diluted CFSE staining to a comparable degree to the IgG and IgM/G B cells. In three separate experiments there was no difference in the intensity of CFSE staining of the remaining IgM or IgM/G transgenic B cells, simply a difference in the absolute number of cells. This implies that IgM B cells are capable of dividing at least 6 to 8 times and suggests little difference in the overall rate of cell division. Instead, IgM transgenic B cells must undergo a greater rate of loss during clonal expansion. This is not due to migration to the bone marrow or preferential differentiation into AFCs at other sites, as few bone marrow IgM AFCs form during this response (see Figure 4-2, part E). Also, production of serum anti-lysozyme IgM, which is a measure of global antibody production, is not increased by seeding even 10^6 IgM transgenic B cells into a primary anti-lysozyme response (Figure 3-1, parts B and C). The most reasonable explanation for these data is that the IgM transgenic B cells die *in situ*. Thus, expression of the IgG membrane tail does not alter the rate of activation or proliferation in T cell-driven responses, but greatly diminishes the attrition of progeny B cells between day 3 and day 5, during the later phases of clonal expansion. This leads to an increased net clonal burst size, increased production of effector cells and a rapid rise in serum IgG antibody titre.

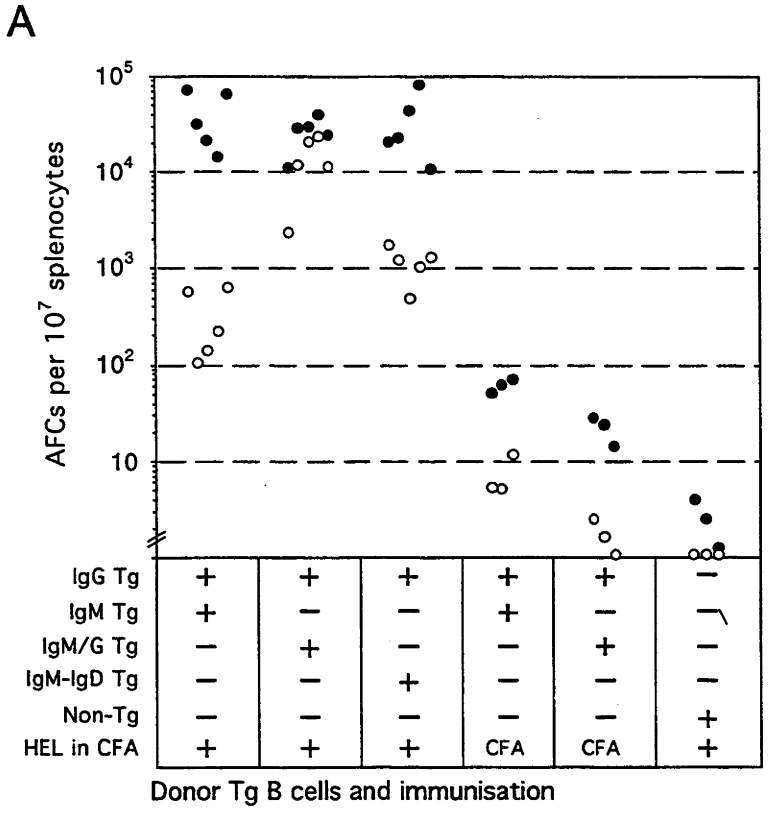
Figure 3-3. The IgG tail augments clonal expansion

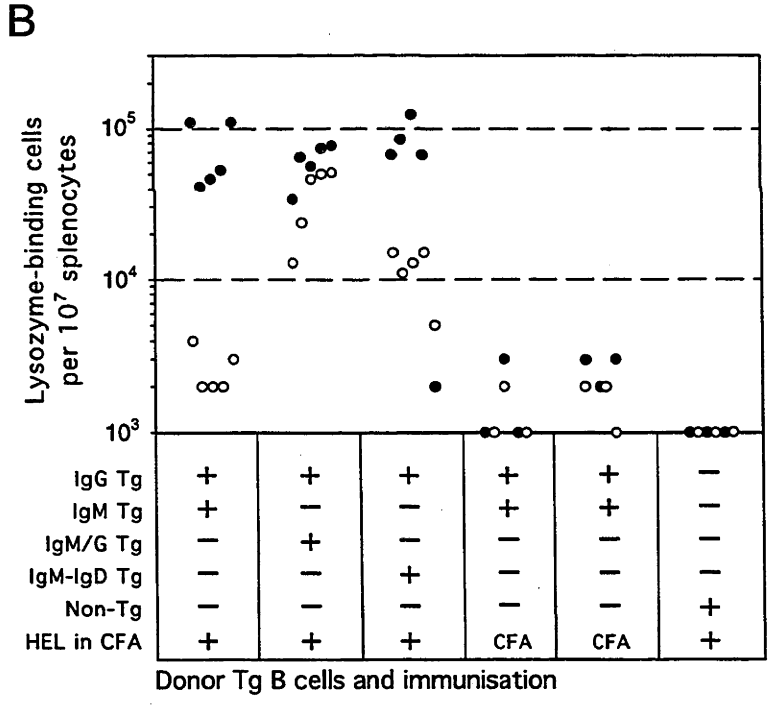
Recipient (B10.BRxC57BL/6)F_1 mice received 10^6 3A9 TCR transgenic $CD4^+$ T cells to increase the number of lysozyme-specific helper T cells, together with mixtures of 10^6 transgenic B cells of the indicated types (i.v.). Recipients were immunised at the same time with lysozyme in adjuvant or adjuvant alone (i.p.).

A. Levels of splenic anti-lysozyme AFCs enumerated by ELISPOT on day 5 after donor cell transfer and immunisation. Anti-lysozyme IgG_1 AFCs in individual recipients are represented by filled circles, anti-lysozyme IgM^a AFCs by open circles.

B. Number of splenic lysozyme-binding B cells in the same animals as (A), enumerated by FACS. Comparable results were obtained in 2 separate co-transfer experiments and in three additional experiments where IgG (GG4), IgM (MM4), IgM/G (MG2) and IgM/IgD (MD4) B cells were transferred singly into separate recipients. Filled circles: IgG transgenic B cells; open circles: IgM, IgM/G and IgM/IgD transgenic B cells.

C. Representative FACS analysis from samples in (B). Staining for cell surface HEL-binding receptors and IgM^a is shown gated on HEL^+ $CD4^-$ lymphocytes from transfer recipients. The percentage of total splenic lymphocytes in each quadrant is given. Details of the FACS gating strategy can be found in 2.7 (c).


A
AFCs per 10^7 splenocytes
10^5
10^4
10^3
10^2
10
IgG Tg + + + + + −
IgM Tg + − − + − −
IgM/G Tg − + − − + −
IgM-IgD Tg − − + − − −
Non-Tg − − − − − +
HEL in CFA + + + CFA CFA +
Donor Tg B cells and immunisation


B
Lysozyme-binding cells per 10^7 splenocytes
10^5
10^4
10^3
IgG Tg + + + + + −
IgM Tg + − − + + −
IgM/G Tg − + − − − −
IgM-IgD Tg − − + − − −
Non-Tg − − − − − +
HEL in CFA + + + CFA CFA +
Donor Tg B cells and immunisation

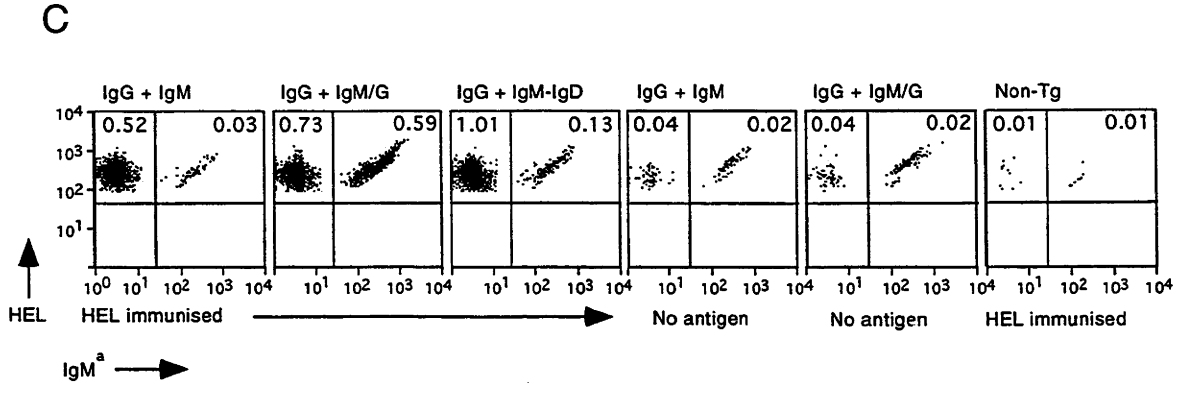

C
IgG + IgM
IgG + IgM/G
IgG + IgM-IgD
IgG + IgM
IgG + IgM/G
Non-Tg
0.52 0.03
0.73 0.59
1.01 0.13
0.04 0.02
0.04 0.02
0.01 0.01
HEL
HEL immunised
No antigen
No antigen
HEL immunised
IgM^a

Figure 3-4. All transgenic lines expressing the IgG membrane tail make an equivalent response to antigen *in vivo*

Recipient (B10.BRxC57BL/6)F_1 mice were immunised with HEL in CFA (i.p.), and at the same time received 10^6 TCR transgenic T cells and 10^6 of the indicated type of transgenic B cell (i.v.).

A. ELISPOT data from day 5 after transfer and immunisation demonstrating the equivalent behaviour of transgenic lines expressing the IgG membrane tail (MG2, MG6 and MG8) compared to lines expressing IgM alone (MM-4) or IgMδE (MδE1 and MδE2). This graph shows the levels of lysozyme-specific IgG_1 (filled circles) and IgM^a (open circles) AFCs in the spleens of transfer recipients, expressed as the number of AFCs per 10^7 total lymphocytes.

B. Clonal expansion data showing lysozyme-binding cell numbers per 10^7 total lymphocytes from the same mice described in (A). Again all the transgenic lines bearing the IgG membrane tail made an ~10-fold greater clonal expansion response compared to transgenic lines bearing IgM or IgD. Note single "non-responding" mice in MG2 and MG6 groups. These may represent mice that received a sub-optimal i.p. injection of HEL/CFA due to puncture of the gut or bladder.

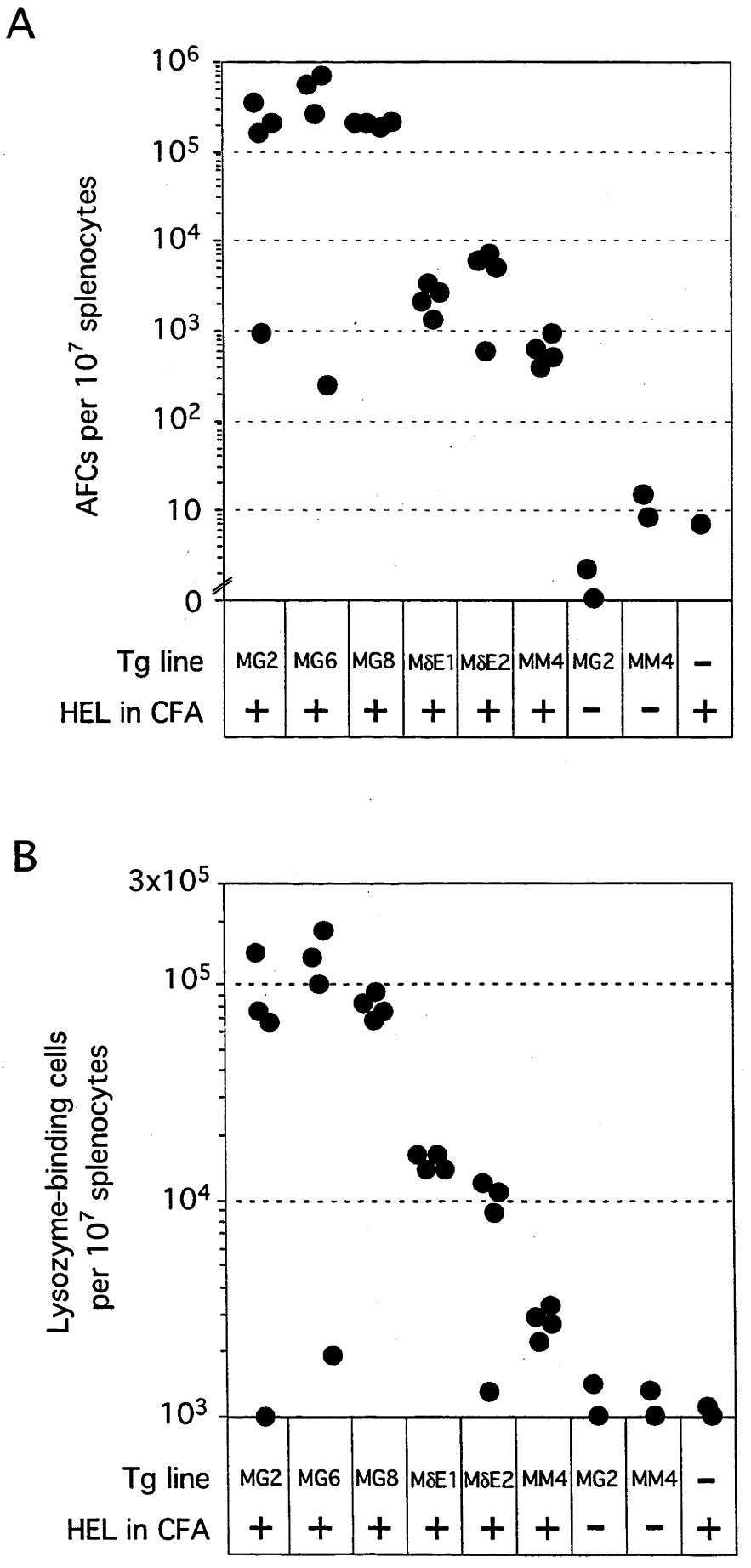
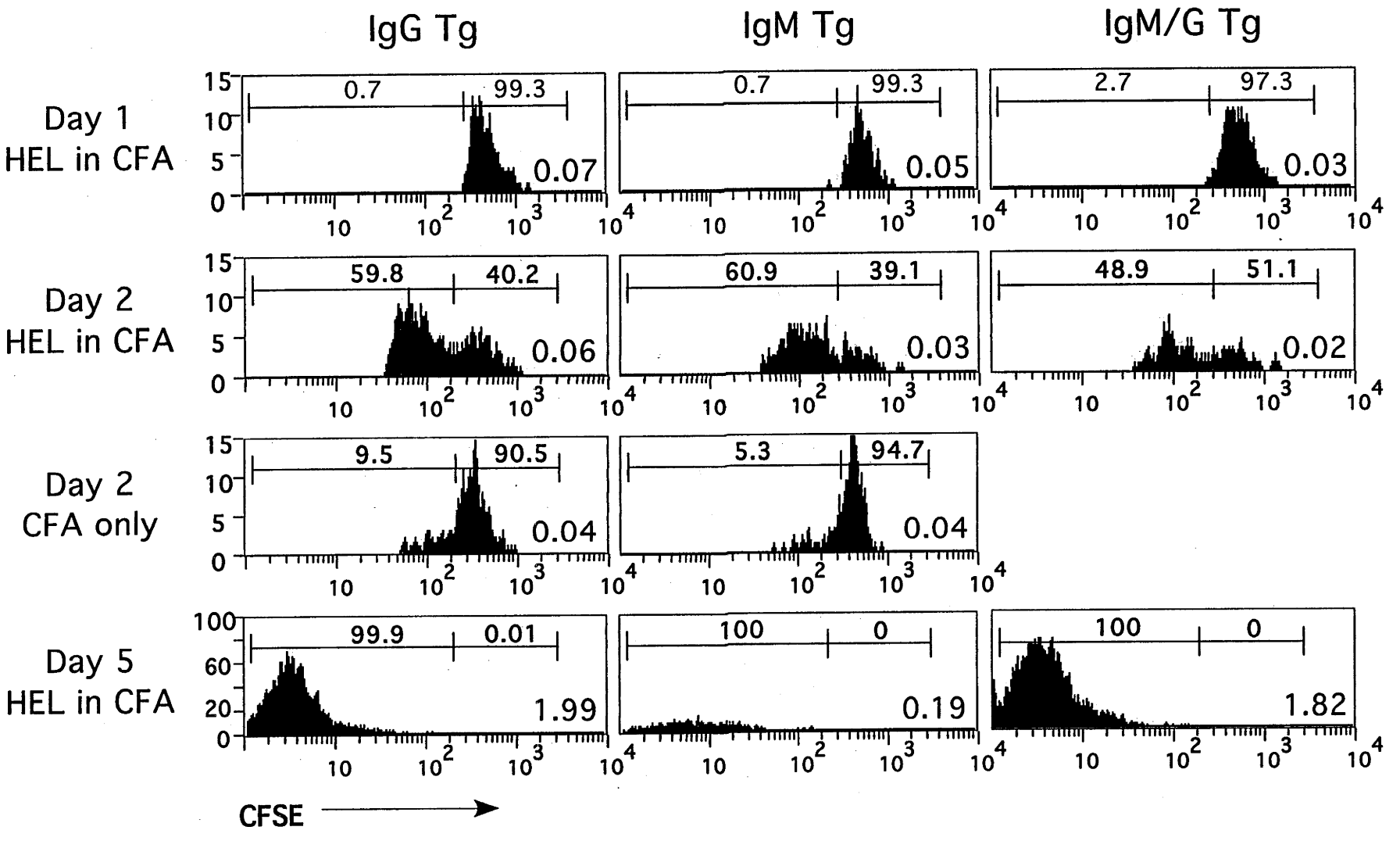

A
AFCs per 10^7 splenocytes
10^6
10^5
10^4
10^3
10^2
10
0
Tg line
HEL in CFA
MG2
MG6
MG8
MδE1
MδE2
MM4
MG2
MM4
–
+
+
+
+
+
+
–
–
+
B
Lysozyme-binding cells per 10^7 splenocytes
3x10^5
10^5
10^4
10^3
Tg line
HEL in CFA
MG2
MG6
MG8
MδE1
MδE2
MM4
MG2
MM4
–
+
+
+
+
+
+
–
–
+

Figure 3-5. IgG, IgM/G and IgM transgenic B cells enter cell division at the same time yet undergo different net clonal expansion during a T-dependent response to antigen *in vivo*

Ig transgenic donor spleen cells were labelled with CFSE and 10^6 IgG, IgM or IgM/G lysozyme-binding B cells transferred per (B10.BRxC57BL/6)F_1 recipient mouse. Each recipient also received 10^6 TCR transgenic $CD4^+$ T cells and was immunised with lysozyme in CFA or CFA alone. Recipient spleen cells were analysed by FACS on day 1, day 2 and day 5 after immunisation to enumerate lysozyme-binding B cells and to measure cell division by the dilution of CFSE. For gating strategies to detect HEL-binding B cells, see 2.7 (c). Histograms show CFSE staining (x-axis) versus cell number, gated on lysozyme-binding B cells of the indicated types. Numbers above gates indicate the percentage of divided or undivided cells amongst the lysozyme-binding B cells. Numbers in the lower right corner show the percentage of lysozyme-binding B cells in the original lymphocyte gate.


IgG Tg
IgM Tg
IgM/G Tg
Day 1
HEL in CFA
Day 2
HEL in CFA
Day 2
CFA only
Day 5
HEL in CFA
0.7
99.3
0.07
0.7
99.3
0.05
2.7
97.3
0.03
59.8
40.2
0.06
60.9
39.1
0.03
48.9
51.1
0.02
9.5
90.5
0.04
5.3
94.7
0.04
99.9
0.01
1.99
100
0
0.19
100
0
1.82
CFSE

Section 3.6. Chapter summary

The basis for heightened secondary antibody responses has been a longstanding unanswered issue confounded by the normally connected differences in specificity, affinity, frequency, priming, and developmental status of antigen-binding B cells in naïve or primed individuals. This study has tracked the *in vivo* immune response of B cells from Ig-transgenic mice using homogeneous populations of B cells bearing a uniform, well-characterised, high-affinity antigen receptor. The BCR only differs within the constant region segments (IgM versus IgG) or only in the carboxy-terminal membrane tail (IgM/G). These results show that IgG BCRs confer a greatly increased clonal burst during the extrafollicular antibody response driven by T cells, and show that the unique membrane tail of IgG is the sole determinant of this heightened burst. The cellular basis for heightened response does not lie in any difference in the activation or rate of proliferation, but is brought about by decreasing the attrition of progeny cells from the later phases of the response.

It is often assumed that immunological memory in antibody responses requires the long-term maintenance of an enlarged pool of antigen-specific B cells (Burnet, 1959). The findings here indicate that an enlarged population of antigen-specific B cells is not sufficient to confer a heightened memory antibody response to a T cell-dependent antigen. Instead, the presence of small numbers of antigen-specific B cells that bear IgG receptors is sufficient for the heightened antibody response, because the unique tail of membrane IgG triggers a greatly increased proliferative burst when the B cell encounters antigen and helper T cells. These findings explain a number of earlier observations. First, the greater proliferative burst of IgG cells provides a mechanism for the dominance of IgG isotypes in memory responses to T-dependent antigens, and for the observation that depletion of IgG bearing B cells from primed lymphocyte mixtures removes most of the memory potential (Coffman, 1977; Yuan, 1977), despite the paradoxically low numbers of isotype-switched B cells present normally in mice and humans (Gray, 1993). The results presented here also explain the augmented ability of IgG bearing cells to produce AFCs upon *in vitro* stimulation (Yefenof, 1986).

These findings are also consistent with previous studies showing the ten-fold lowering of antigen-specific IgG or IgE responses in knockout mice with truncated IgG or IgE tails lacking the cytoplasmic segment (Achatz, 1997; Kaisho, 1997). The findings from the tail-truncation mice established that an intact cytoplasmic segment is important for IgG-bearing B cells, but failed to establish whether the intact cytoplasmic sequence is needed for correct surface expression of IgG or for some specific process during the formation,

survival or response of IgG^+ cells. IgG^+ B cells lacking the cytoplasmic segment exhibited lower surface Ig expression on LPS blasts *in vitro* but expression appeared normal on the few cells that could be detected *in vivo* (Kaisho, 1997). This raises the possibility that the reduced numbers of IgG^+ cells and IgG serum antibody in tail-truncation mice resulted from reduced stability of surface IgG expression. The transgenic approach taken here avoids these problems by directly comparing the function of IgM and IgG in its normal structure, and by adding the complete IgG membrane tail segments to IgM at the natural junction point between the secreted and membrane Ig proteins.

The downstream biochemical mechanism for augmenting B cell clonal expansion through the IgG membrane tail will be of future interest. Some reports have suggested that the YXXM sequence within the IgG cytoplasmic tail domain acts as an endocytosis signal of the YXXϕ type (where ϕ is any bulky, hydrophobic amino acid) to increase antigen presentation through the MHC Class II pathway (Achatz, 1997; Kaisho, 1997; Nussenzweig, 1997), perhaps with a reduced dependence for accessory signaling molecules (Knight, 1997). We cannot rule out the IgG membrane tail playing a role in this process, although we find no evidence that the priming of IgG transgenic cells is more efficient than IgM transgenic cells during their initial activation *in vivo*. It is probable that the IgG membrane tail has other signaling functions, specifically in promoting cell survival during successive cell divisions. It is interesting that the burst-enhancing effect of the IgG membrane tail parallels the burst-enhancing effect of CD28 costimulation or CTLA4-deficiency in helper T cell proliferation (Gudmundsdottir, 1999; Doyle, 2001). In each case, a major mode of action is to decrease the attrition of progeny cells to yield many more effector cells. Collectively, these results underscore the control of cell loss rather than cell proliferation as a key regulatory point for controlling the magnitude of immune responses *in vivo*.

Chapter 4. An examination of factors which affect transgenic B cell responses to antigen *in vivo*

Section 4.1. Introduction

In the previous chapter the IgG membrane tail was shown to have a unique, isotype-specific function *in vivo*. Transgenic B cells expressing the membrane tail of IgG were shown to produce a more robust response compared to IgM transgenic B cells and the effect was due to greater net clonal expansion during a T-dependent immune response. This was not due to more efficient activation of B cells bearing the IgG membrane tail, as the initial entry into cell cycle on day 2 after immunisation was similar in all transgenic B cells tested. By day 5 after immunisation, however, there was a large net clonal expansion of IgG and IgM/G transgenic cells, while very few IgM transgenic cells could be detected by FACS.

This chapter will address the location of the transgenic cells during the immune response in a more detailed study of the events between day 2 and day 5 after immunisation, during a primary anti-lysozyme response in the presence of transgenic T cell help. Also, possible explanations will be explored for the large difference in behaviour between the transgenic B cells expressing or lacking the IgG membrane tail.

Section 4.2. The location and phenotype of IgG and IgM transgenic B cells during an immune response

For experiments described in Section 4.2, 10^6 IgM/G or 10^6 IgM transgenic B cells were seeded along with 10^6 TCR transgenic T cells into lysozyme-immunised recipient mice (for details see legends to Figure 4-1, Figure 4-2).

4.2 (a) Histology

In order to follow the fate of the transgenic B cells in the time period between days 2 and 5, spleen sections were cut on days 3, 4 and 5 after immunisation and transfer from recipients that received IgM/G or IgM transgenic B cells (Figure 4-1). By staining for HEL-binding cells, syndecan-binding cells and B cell follicles it was possible to follow the expansion, location and differentiation of the transferred transgenic B cells. The results are consistent with a predominantly extrafollicular response for both IgM/G and IgM transgenic B cells during a primary response to lysozyme in the presence of transgenic T cell help.

In both cases IgM/G and IgM transgenic B cells are syndecan negative on day 3, and were found in small perifollicular foci, often near the bridging channels which connect the T zone and red pulp (Figure 4-1, parts A→D). On day 4, in the case of IgM/G recipients, a few transgenic cells had started to migrate *via* bridging channels to the red pulp, although the majority were still syndecan negative. For both IgM/G and IgM recipients, transgenic cells on day 4 were in perifollicular areas, often forming a distinctive ring around the edge of B cell follicles. There seemed to be fewer IgM transgenic B cells compared to IgM/G transgenic B cells in the three mice of each type analysed at this timepoint (Figure 4-1, parts E→H).

On day 5 there were large differences in the extent of transgenic B cell net clonal expansion between IgM and IgM/G transgenic recipients (Figure 4-1, parts I, J). In the case of IgM/G transgenic cells, small foci were no longer apparent and the bridging channels and the T zones were filled with HEL-binding cells that were syndecan positive. This may have represented the transition from small extrafollicular foci to dispersed red pulp plasma cells described previously in the NP system (Sze, 2000). In the case of IgM recipients, HEL-binding syndecan-positive cells were visible in very few areas of the spleen in similar locations (see Figure 4-3, panels K, L). In most regions however, there were very few HEL-binding IgM transgenic B cells. They were no longer in perifollicular foci had probably died *in situ*.

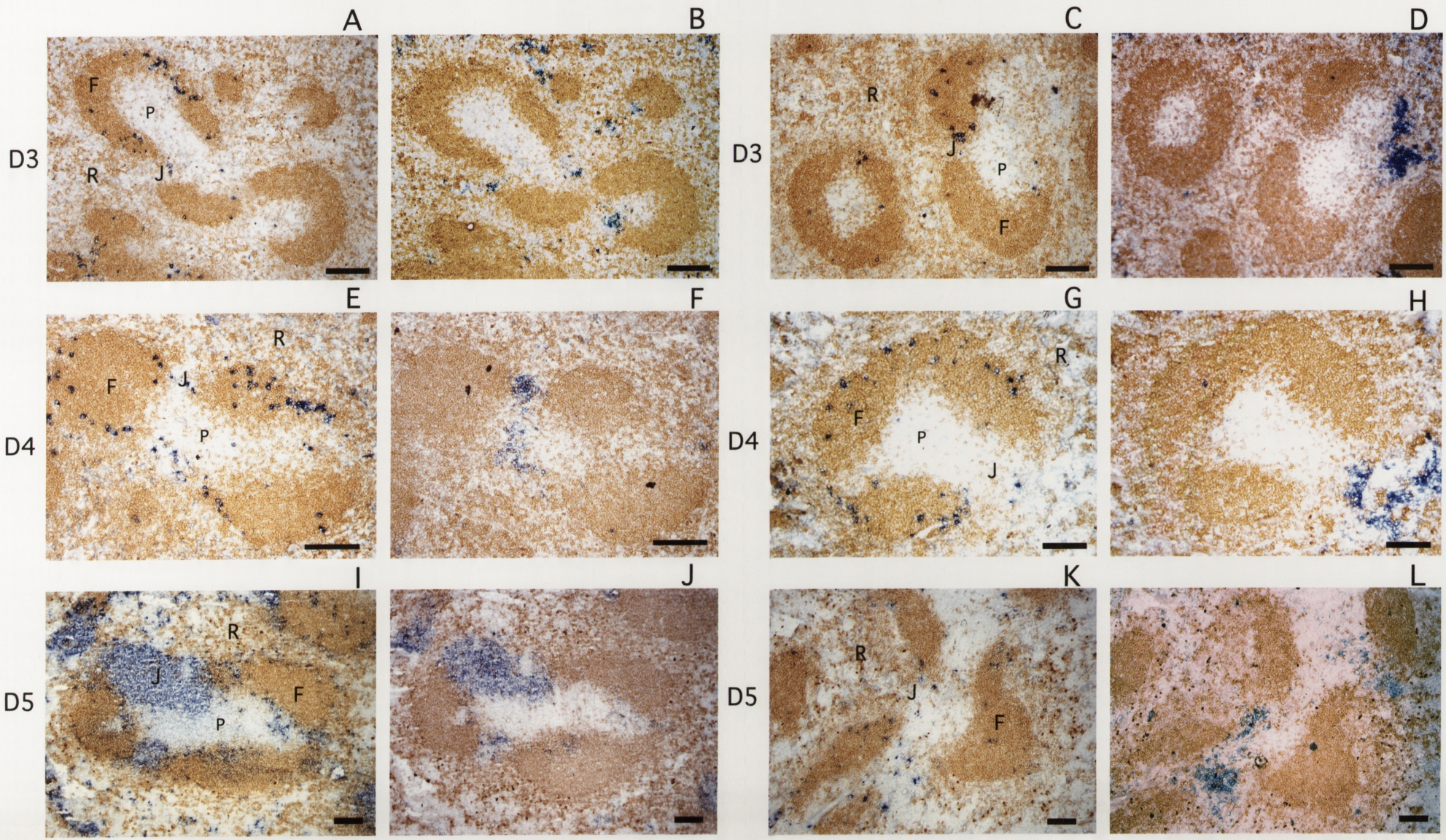
Reports using the NP hapten system have suggested that extrafollicular plasmablast foci are not sustained by the presence of antigen-specific T cells (Han, 1995a; Gulbranson-Judge, 1996), but rather associate with CD11c^{hi} dendritic-morphology cells (Garcia de Vinuesa, 1999). In addition, the relative number of these plasmablast-associated dendritic cells (PDCs) may be an extrinsic limiting factor determining plasma cell numbers in the spleen (Sze, 2000). Translating those observations to this system, it may be that the IgG membrane tail makes IgM/G transgenic B cells less dependent on limiting survival signals provided by PDCs within extrafollicular foci. Alternatively, the IgG membrane tail may protect IgM/G transgenic B cells from death-inducing signals during exposure to a highly cross-linking form of HEL, possibly immune complex held on the surface of PDCs (see section 4.2 (b)).

The spleens of transfer recipients that received IgM/G or IgM transgenic cells showed no evidence of germinal centre or follicular expansion by day 5. Under this immunisation protocol, both IgM and IgM/G transgenic cells were induced to expand and differentiate in extrafollicular sites. Experiments to investigate the follicular/GC pathway of expansion and differentiation will be decribed in more detail in Section 4.3.

Figure 4-1. Histological analysis of IgM, IgM/G or non-transgenic recipient spleens on day 3, day 4 and day 5 after immunisation and transfer.

10^6 IgM/G (A, B, E, F, I, J) or 10^6 IgM (C, D, G, H, K, L) donor transgenic B cells were transferred with 10^6 TCR transgenic T cells into (B10.BRxC57BL/6)F_1 recipients and the animals immunised with HEL in CFA. Recipient spleens were harvested on day 3 (A, B, C, D), day 4 (E, F, G, H) and day 5 (I, J, K, L) after immunisation and B cell transfer and half of the sample frozen in liquid nitrogen.

Serial 5μm frozen sections were prepared and stained as described in materials and methods (Section 2.11) for either HEL-binding cells (A, C, E, G, I, K) or syndecan positive cells (B, D, F, H, J, L) in blue and for IgD to show B cell follicles in brown. Bars represent 150 μm. Markings on the sections indicate the location of follicles (F), red pulp (R), junctional zones (J) and the PALS (P).


IgM/G Tg
IgM Tg
A
B
C
D
E
F
G
H
I
J
K
L
D3
D3
D4
D4
D5
D5
F
P
R
J

4.2 (b) FACS

In addition to locating the transferred transgenic B cells on days 3, 4 and 5 after immunisation, it was important to track their expansion by FACS. Cell division progression was investigated on days 3, 4 and 5 after transfer by loading IgM/G and IgM transgenic B cells with a higher concentration of CFSE (10 μM compared to 2.5 μM) prior to transfer, to try to increase the range of cell division cycles that could be measured.

On day 3 after immunisation and transfer, HEL-binding B cells in both IgM/G and IgM recipients could be located as a small on-diagonal population when stained for HEL using two fluorochromes simultaneously (Figure 4-2, part A). The CFSE profiles of the detectable HEL-binding cells revealed that both IgM/G and IgM transgenic cells had undergone a similar number of cell division cycles (~4-6) by day 3 (Figure 4-2, part B). This implies that IgM and IgM/G transgenic cells have similar rates of cell division up to day 3 after immunisation and transfer.

On day 4 after immunisation and transfer, HEL-binding B cells of the IgM/G and IgM transgenic types could not be detected by FACS (Figure 4-2, part A). This was not due to a failure of the staining protocol, as HEL-binding cells were clearly visible in a positive control containing "spiked" IgM/G transgenic B cells that were added just prior to staining. Also, ELISPOT measurements on samples from day 4 suggested that HEL-specific AFCs were present at this timepoint (Figure 4-2, part C), and both IgM and IgM/G transgenic B cells were visible by histology in sections at this timepoint (Figure 4-1, panels I→L). The FACS staining method relies on detecting the anti-HEL BCR, suggesting that transgenic B cells strongly modulate surface Ig expression at this timepoint. The phenomenon of receptor modulation *in vivo* during a T-dependent response has been noticed by others using the anti-HEL MD4 line (Garside, 1998). In the present study, modulation may have reflected exposure to a multivalent form of HEL, perhaps as immune complex between low-affinity endogenous anti-HEL IgM produced early in the response and HEL presented by FcR-mediated binding on the surface of dendritic cells. If this is correct, it may offer a mechanism for the dramatic loss of IgM transgenic cells after this timepoint, as exposure of IgM transgenic cells to this modulating form of antigen may have induced cell death, while transgenic B cells expressing the IgG membrane tail were protected.

By day 5 after immunisation and transfer, both IgM/G and IgM transgenic B cells could be detected by FACS using stains directed against the BCR (Figure 4-2, part A), however there was a large, >10-fold difference in net clonal expansion. The ability to

visualise HEL-binding cells by FACS correlated with the timepoint at which IgM/G and IgM transgenic cells were no longer in perifollicular foci, but were located near the bridging channels and red pulp.

The expansion rate of IgM/G transgenic B cells at extrafollicular sites between days 3 and 5 is very rapid, as estimated by FACS. ~20,000 IgM/G transgenic B cells could be enumerated per spleen on day 3 and these gave rise to ~2,000,000 cells per spleen on day 5 (Figure 4-2, part D). Even in the absence of cell death, the cell division cycle time on the basis of these numbers must have been ~ 8 hours. Certainly there is precedence for cells of the B lineage to undergo very rapid cell division of the order of ~6-7 hours per cycle in the specialised microenvironment of the germinal centre dark zone (Zhang, 1988; Liu, 1991). Estimates for cell division cycle time during plasmablast expansion in non-GC extrafollicular sites during a primary response vary, but may be of the same order. Jacob and Kelsoe (Jacob, 1991) estimated a 16-20 hour division time in foci during the primary NP response, but Sze *et al.* (Sze, 2000) and Toellner *et al.* (Toellner, 1996) showed that plasmablast expansion could proceed with a cell cycle time closer to 10-14 hours. This at least shows that the estimates for cell division rate presented here for extrafollicular plasmablasts on the basis of FACS enumeration are plausible.

An alternative possibility is that the day 3 FACS enumeration is an underestimate, perhaps due to BCR modulation masking the transgenic B cells, as described above. In the future it may be possible to use non-BCR markers such as CD45 allotypes to track the transgenic B cells, to see if there are discrepancies between the actual number of cells present and the number of cells that can be detected during the clonal expansion process by staining for BCR expression.

4.2 (c) AFC production

As well as the location and expansion of Ig transgenic B cells over the course of the immune response, their differentiation into AFCs was measured by ELISPOT. AFC production in the spleen was assessed on days 3, 4 and 5 after immunisation and transfer in both IgM and IgM/G recipients (Figure 4-2, part C). By this assay, the number of anti-HEL IgM^a-secreting cells increased steadily from day 3 through day 5 for both the IgM and IgM/G transgenic recipients. This assay had the advantage that it did not rely on detecting transgenic cells by the expression of high levels of BCR. Clearly there was a quantitative difference in the production of AFCs between transgenic lines, with approximately 100-fold more IgM/G derived AFCs on day 4 and day 5 compared to IgM AFCs.

Interestingly, transgenic cell enumeration by FACS and AFC numbers by ELISPOT agreed very well for both IgM and IgM/G recipients on day 5, suggesting the majority of transgenic cells were at some stage of plasmacytoid differentiation.

This result provides further evidence that IgM transgenic B cells are capable of differentiating to antibody-secreting cells, as there are nearly 1000-fold more IgM-derived AFCs on day 5 compared to day 3. Despite this, the IgM transgenic B cells consistently produce fewer AFCs compared to the IgM/G transgenic B cells. One possible explanation could be preferential migration and plasma cell differentiation of IgM transgenic B cells in the bone marrow, an important site of long-term antibody production (Benner, 1981; Slifka, 1998a). This was ruled out by measuring the anti-HEL IgM^a AFC response in recipient bone marrow on day 5 after immunisation and transfer of IgM/G or IgM transgenic cells (Figure 4-2, part E). There were very few AFCs in the bone marrow of either IgM/G or IgM transgenic recipients at day 5, suggesting plasma cell migration to the bone marrow cannot explain the differences in spleen clonal expansion or AFC production.

4.2 (d) Summary

These experiments showed that there are few qualitative differences in the response of IgM/G and IgM transgenic B cells during a primary immune response to HEL. Neither IgM/G nor IgM transgenic B cells were located in follicular or germinal centre sites during this response, nor was there significant formation of anti-HEL AFCs in the bone marrow. Rather, both types of transgenic B cell followed an exclusively extrafollicular pathway of expansion and differentiation. They were present in small extrafollicular foci on day 3 and those cells detectable by FACS had undergone an equivalent number of cell division cycles. The most striking differences manifested on days 4 and 5, as the transgenic B cells underwent a transition from extrafollicular foci of syndecan-negative cells to become more dispersed syndecan-positive plasmablasts that fill the bridging channels and red pulp. On day 4 after transfer there was a striking down-modulation of IgM and IgM/G BCRs, possibly due to exposure to multivalent HEL in immune complex. The large quantitative difference between IgM and IgM/G transgenic B cell expansion on day 5 by both FACS and histology suggests that the protective effect of the IgG membrane tail manifests late in the clonal expansion process.

Figure 4-2. Timecourse of expansion by transferred IgM and IgM/G transgenic B cells and AFC production in the spleen and bone marrow

10^6 CFSE-labelled IgM or IgM/G transgenic or non-transgenic B cells were transferred along with 10^6 TCR transgenic T cells into (B10.BRxC57BL/6)F_1 recipients immunised at the same time with HEL in CFA.

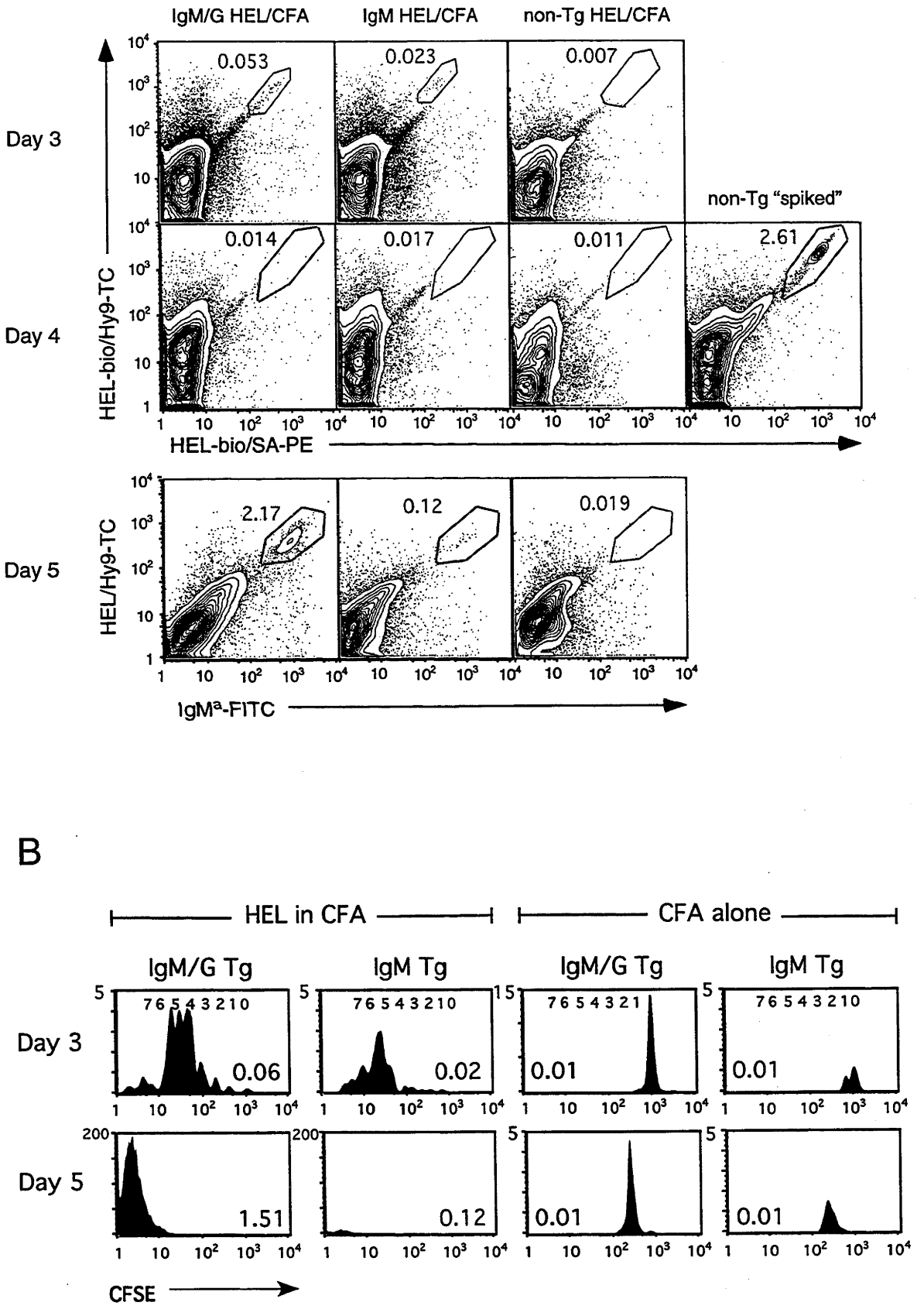
A. Representative FACS plots of IgM/G and IgM transgenic B cells showing the FACS enumeration of HEL-binding cells on days 3, 4 and 5 after transfer. Transgenic B cells were stained for transgenic BCR on two channels simultaneously, and form a tight on-diagonal population. These contour plots are gated on $CD4^-$ lymphocytes and show the gate used to detect HEL-binding cells. The number refers to the cells falling in the HEL-binding gate and is given as a fraction of total splenic lymphocytes.

B. CFSE histograms of IgM/G and IgM transgenic B cells in recipient spleen suspensions on days 3 and 5 after immunisation with HEL/CFA or CFA alone. These histograms were gated on HEL-binding $CD4^-$ lymphocytes, as shown in (A). The numbers within the plots refer to the fraction of total splenic lymphocytes that the cells represent. Also on day 3 histograms the small numbers show approximate cell division cycle numbers, based on the position of the undivided peaks in the CFA immunised samples.

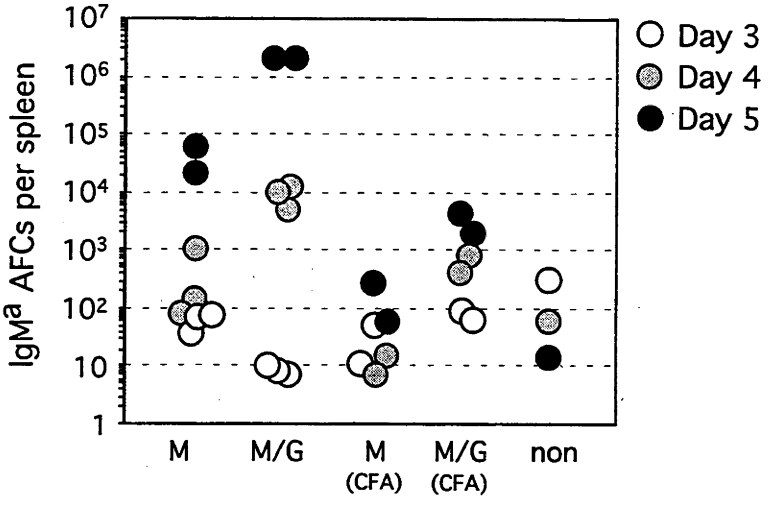
C. Graph showing anti-lysozyme IgM^a AFC production on days 3 (open circles), 4 (shaded circles) and 5 (filled circles) by IgM/G and IgM transgenic B cells. Data is given as total AFCs per recipient spleen.

D. Graph showing the net clonal expansion of IgM and IgM/G transgenic B cells on days 3 (open circles) and 5 (filled circles) enumerated by FACS as described in (A). Data is given as total HEL-binding cells per spleen.

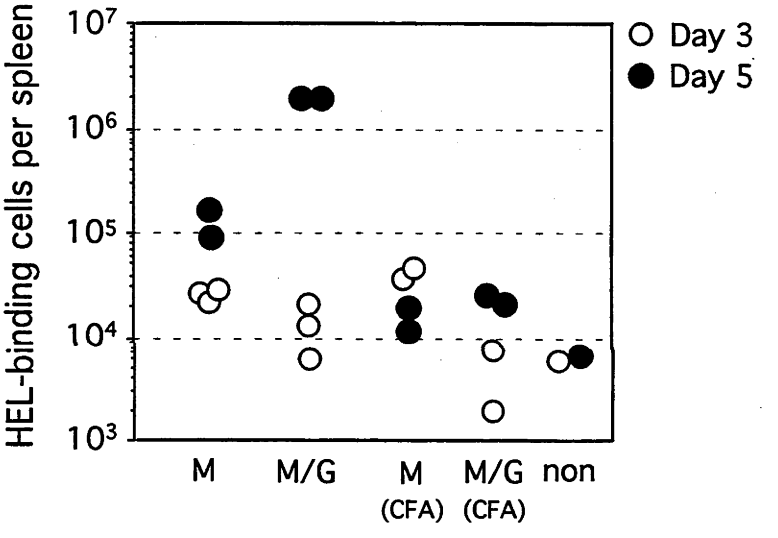
E. Graph showing anti-lysozyme IgM^a AFC production in the spleen and bone marrow of recipients that received IgM/G and IgM transgenic B cells, on day 5 after immunisation and transfer. These data are taken from a separate experiment to (A), (B), (C) and (D), and are included to show the low numbers of anti-lysozyme bone marrow AFCs detected on day 5 of the response.


A
Donor B cells and immunisation
IgM/G HEL/CFA
IgM HEL/CFA
non-Tg HEL/CFA
non-Tg "spiked"
Day 3
Day 4
Day 5
0.053
0.023
0.007
0.014
0.017
0.011
2.61
2.17
0.12
0.019
HEL-bio/Hy9-TC
HEL-bio/SA-PE
HEL/Hy9-TC
IgMa-FITC
B
HEL in CFA
CFA alone
IgM/G Tg
IgM Tg
IgM/G Tg
IgM Tg
Day 3
Day 5
0.06
0.02
0.01
0.01
1.51
0.12
0.01
0.01
CFSE

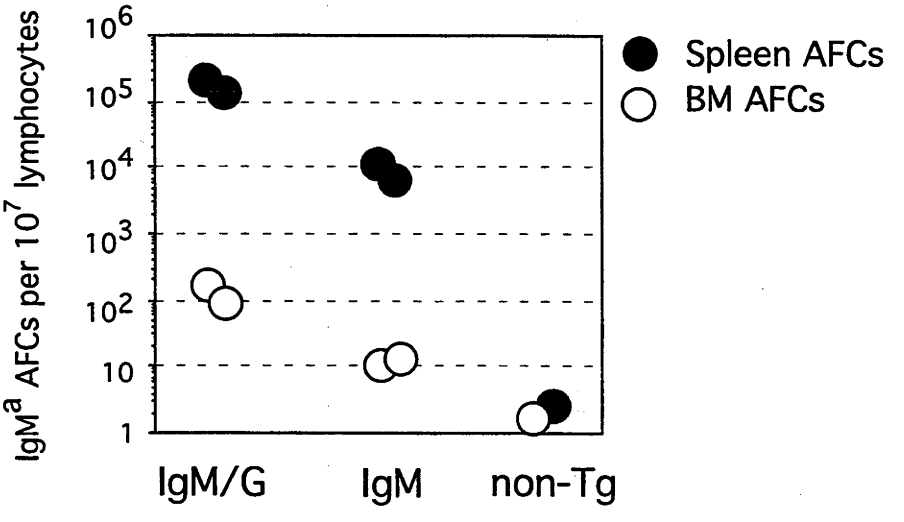
C


IgM^a AFCs per spleen
10^7
10^6
10^5
10^4
10^3
10^2
10
1
M
M/G
M (CFA)
M/G (CFA)
non
Day 3
Day 4
Day 5

D


HEL-binding cells per spleen
10^7
10^6
10^5
10^4
10^3
M
M/G
M (CFA)
M/G (CFA)
non
Day 3
Day 5

E


IgM^a AFCs per 10^7 lymphocytes
10^6
10^5
10^4
10^3
10^2
10
1
IgM/G
IgM
non-Tg
Spleen AFCs
BM AFCs

Section 4.3. Germinal centre and follicular expansion of IgM/G and IgM transgenic B cells

The lack of a follicular or germinal centre responses by both IgM/G or IgM transgenic B cells on day 5 after immunisation and transfer into unprimed recipients was an interesting observation. In a similar system in which antigen-specific transgenic T and B cells were seeded into immunised recipients, antigen-specific germinal centres were noticed in recipient lymph nodes on day 4 after immunisation and transfer (Garside, 1998).

It was important to determine whether the lack of germinal centres in this system was due to the inability of IgM/G and IgM transgenic B cells to participate in germinal centres, or to other factors such as the type and location of T cell help. To test this, recipient mice were primed with antigen and TCR transgenic cells for 6 days prior to the injection of transgenic B cells with a boost of soluble antigen. This was a system that had been used previously to study the consequences of antigen engagement on HEL-specific germinal centre B cells (Shokat, 1995). It was reasoned that priming with antigen in adjuvant would induce normal anti-lysozyme germinal centres, and that transferred transgenic B cells would then participate in the germinal centre reaction.

Three days after the introduction of the transgenic B cells into primed mice, both IgM and IgM/G B cells were dispersed inside the B cell follicles, with no evidence of extrafollicular foci by histology (Figure 4-3, panels A →D). Numerous PNA^+ germinal centres that contained HEL protein staining on follicular dendritic cell networks were visible at this timepoint (Figure 4-3, panels M and N).

By day 5 after the introduction of transgenic B cells into primed mice, very few HEL-binding B cells were located within the B cell follicles by histology (Figure 4-3, panels E→H). HEL-binding B cells seemed to be present in some germinal centres in both IgM and IgM/G recipients. It is not known whether these germinal centres nucleated around the transgenic cells, or whether transgenic B cells were able to move into existing germinal centres. Also, it seems unlikely that all of the transgenic B cells visible at day 3 moved into germinal centres. Many may have died *in situ* between day 3 and day 5, possibly because they were unable to incorporate into already-established germinal centres.

The histological picture of Ig transgenic B cells seeded into primed mice is in direct contrast to the system where Ig transgenic B cells were seeded into unprimed recipients (Figure 4-3, panels I→L). In the absence of priming, no prominent germinal centres were

present, and both IgM/G and IgM transgenic B cells formed exclusively extrafollicular foci of HEL-binding cells.

One explanation for these observations is that the type and location of T cell help is different in the two adoptive transfer systems. It seems likely that in the case where both transgenic B cells and transgenic T cells are naïve upon transfer and immunisation, the high affinity of the Ig BCR leads to synchronous, early activation of most transgenic B cells. The majority of the transgenic B cells may be directed by the early T cell help and abundant soluble antigen to seed extrafollicular foci. This is the fate of many B cells which are activated early during both T-dependent and T-independent immune responses (Liu, 1991; Smith, 1996).

By contrast, in the experimental system where transgenic T cells are primed by antigen in adjuvant for 6 days, observations from the pigeon cytochrome *c* TCR transgenic system suggest that T cell help shifts to be located mostly in B cell follicles (Kearney, 1994; Ansel, 1999). This is probably due to the requirement for T cells to upregulate the chemokine receptor CXCR5 and become responsive to the chemokine CXC13 before they can enter B cell follicles and provide help to B cells to support germinal centres (Ansel, 1999; Breitfeld, 2000; Schaerli, 2000; Kim, 2001). Although direct evidence for primed 3A9 TCR transgenic T cells being located in B cell follicles after 6 days of priming is not available, the fact that transgenic B cells expand exclusively in the B cell follicles or primed mice is suggestive of the fact. In the future it may be possible to use the clonotypic antibody 1G12 to investigate the location of the TCR transgenic T cells histologically during a primary response and after 6 days of antigen priming.

Section 4.4. IgM transgenic B cells do not undergo preferential apoptosis *via* FasL expressed on transgenic helper T cells

When IgM and IgM/G transgenic B cells were seeded into unprimed recipients in the presence of antigen and naïve T cell help they were activated to a similar degree (Figure 3-5). In addition, the rate of proliferation of IgM and IgM/G transgenic B cells was similar, yet there was a profound difference in net clonal expansion (Figure 4-2, parts A, B and E). Net clonal expansion is a balance between the rate of cell proliferation and the rate of cell death, raising the likelihood that IgM transgenic cells undergo a greater rate of death during successive cell divisions.

One candidate pathway for cell death that is important for both lymphocyte tolerance (Rathmell, 1994; Rathmell, 1995; Goodnow, 1996; Rathmell, 1996) and as a brake on T

cell immune responses (van Parijs, 1998) is mediated by the TNF/TNFR family member Fas (CD95). Fas is expressed by activated B cells and FasL is expressed by activated T cells. Only acute antigen stimulation through the BCR saves naïve B cells from elimination *via* this pathway (Rathmell, 1996), chronic antigen exposure in anergic cells leads to Fas-mediated apoptosis. There is some evidence that memory and naïve B cells may be regulated differentially *via* this pathway, as while memory B cells express low levels of Fas, they have been reported to be resistant to cell death *via* Fas ligation (Liu, 1995).

To test whether IgG or IgM transgenic B cells were more or less sensitive to Fas-mediated apoptosis induced by transgenic helper T cells during an immune response *in vivo*, IgM and IgG transgenic B cells were transferred into HEL-immunised recipient animals along with normal TCR transgenic cells or TCR transgenic cells carrying the $FasL^{gld}$ mutation. The $FasL^{gld}$ phenotype is due to a mutant FasL on transgenic T cells that is unable to bind Fas expressed by naïve IgM or IgG transgenic B cells (Goodnow, 1995). It was reasoned that under these conditions, Fas-mediated transgenic T cell killing of IgM transgenic cells would be absent, so IgM and IgM/G transgenic B cells should show similar clonal expansion and antibody production if this pathway was solely responsible for the observed differences (Figure 4-4).

Figure 4-3. Follicular and germinal centre expansion of IgM and IgM/G transgenic B cells in primed recipients

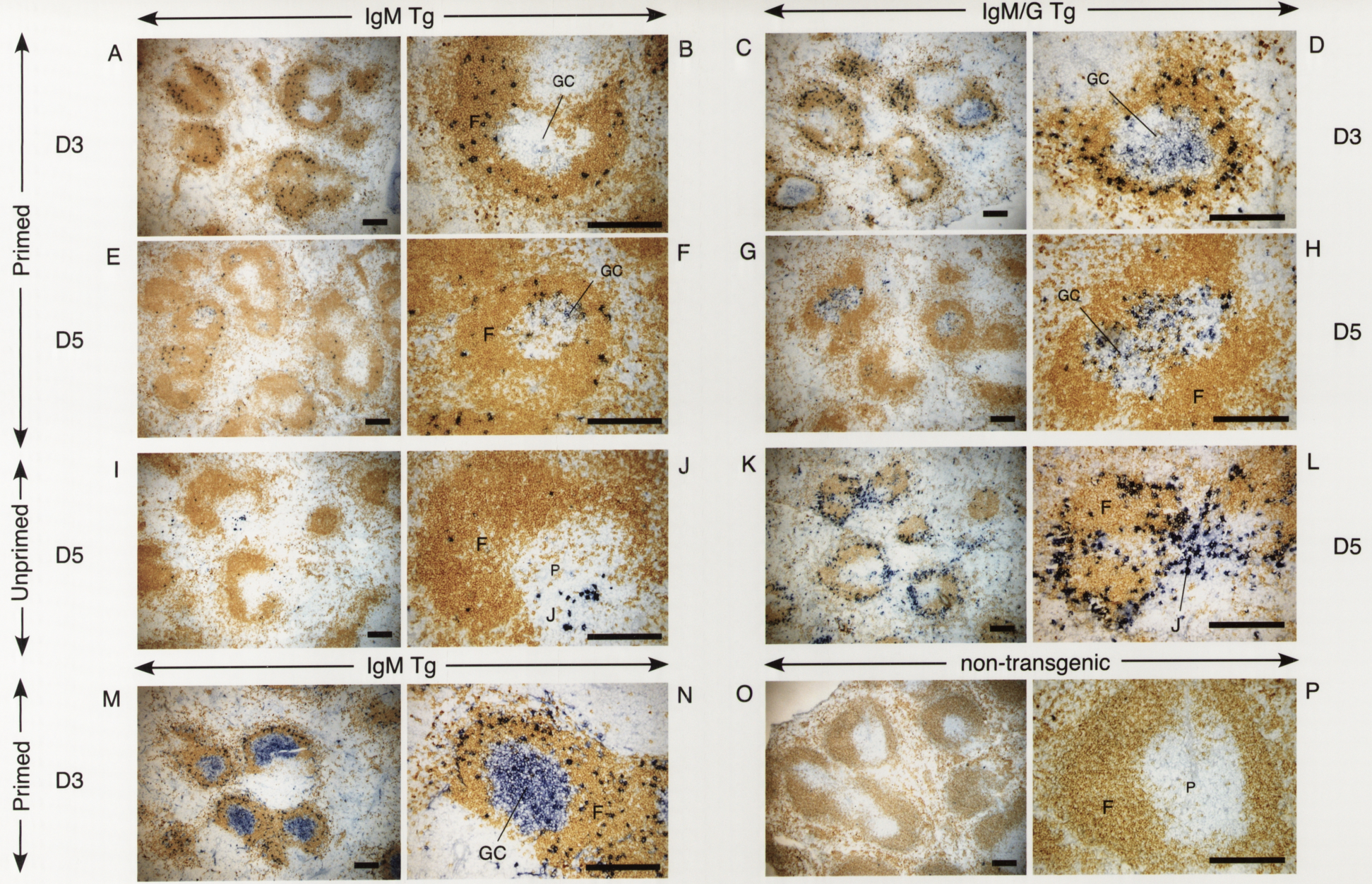
Histology of recipient mouse spleen sections on days 3 and 5 after transfer of IgM transgenic (panels A, B, E, F, I, J, M, N), IgM/G transgenic (C, D, G, H, K, L) or non-transgenic (O, P) B cells.

"Primed" recipient (B10.BRxC57BL/6)F_1 mice were given 10^6 TCR transgenic cells and HEL in CFA 6 days prior to the transfer of 10^6 transgenic B cells with 50 μg boosting soluble antigen and isoproterenol (panels A→H, M, N, O, P).

"Unprimed" recipients (panels I→L) were treated as described previously in Figure 4-1.

5 μm frozen sections were prepared, and B cell follicles stained for IgD (brown) and HEL-binding (blue), or PNA and HEL-binding together (panels M and N). Bars represent 200 μm. Note the lack of HEL-binding cells in non-transgenic recipient spleens that were immunised with HEL/CFA (panels O and P), demonstrating that only B cells bearing transgenic anti-lysozyme receptors are stained by this protocol.

Labels on the sections indicate germinal centres (GC), B cell follicles (F), PALS (P) and junctional zones (J).


IgM Tg
IgM/G Tg
non-transgenic
Primed
Unprimed
Primed
D3
D5
D5
D3
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
GC
F
P
J

Clearly, even when TCR:*FasL*^{gld} transgenic T cells are co-transferred with IgM and IgM/G transgenic B cells there is still a 10-100-fold greater net clonal expansion of IgM/G transgenic cells compared to IgM transgenic cells (Figure 4-4, part A). Interestingly, the level of clonal expansion and especially AFC production in both IgM and IgM/G transgenic recipients was decreased when TCR:*FasL*^{gld} H-2^{kk} helper cells were used compared to normal FasL expressing TCR H-2^{kk} transgenic cells (Figure 4-4, part B). This is in accordance with previous observations that Fas/FasL contact can provide a mitogenic signal during acute B cell activation with antigen (Rathmell, 1996).

This experiment contains a number of controls. Firstly, a similar level of clonal expansion was seen when TCR H-2^{kk} transgenic cells were used as helpers compared to TCR H-2^{kb} helpers (see Figure 3-3), showing thatTCR H-2^{kk} helpers were able to provide adequate T help to the H-2^{kb} Ig transgenic cells. Secondly the dependence of this response on contact between antigen-specific TCR and Ig transgenic cells was highlighted by the fact that in the absence of transferred transgenic T cells, both IgG and IgM transgenic B cell responses were significantly depressed.

This experiment demonstrated that the Fas/FasL-mediated apoptosis induced by transgenic T cell help is not responsible for the differences in net clonal expansion between IgM and IgG transgenic B cells, as crippling signalling *via* FasL on transgenic T cells does not rescue clonal expansion or antibody production by IgM transgenic B cells. This is not surprising, given the finding that antigen-specific CD4$^+$ T cells do not localise to extrafollicular foci (Jacob, 1991; Gulbranson-Judge, 1996), the major site of IgM transgenic B cell loss in this system. It remains possible that other recipient components (T cells, DCs) are responsible for the apoptotic loss of IgM transgenic B cells *via* Fas/FasL or some other pathway. To formally exclude other sources of Fas/FasL-mediated killing, the adoptive transfer could be performed in *FasL*^{gld} recipients. In addition, it should be possible to test the role of apoptosis in general in the loss of IgM transgenic B cells by over-expressing an anti-apoptotic factor such as Bcl-2 in that line.

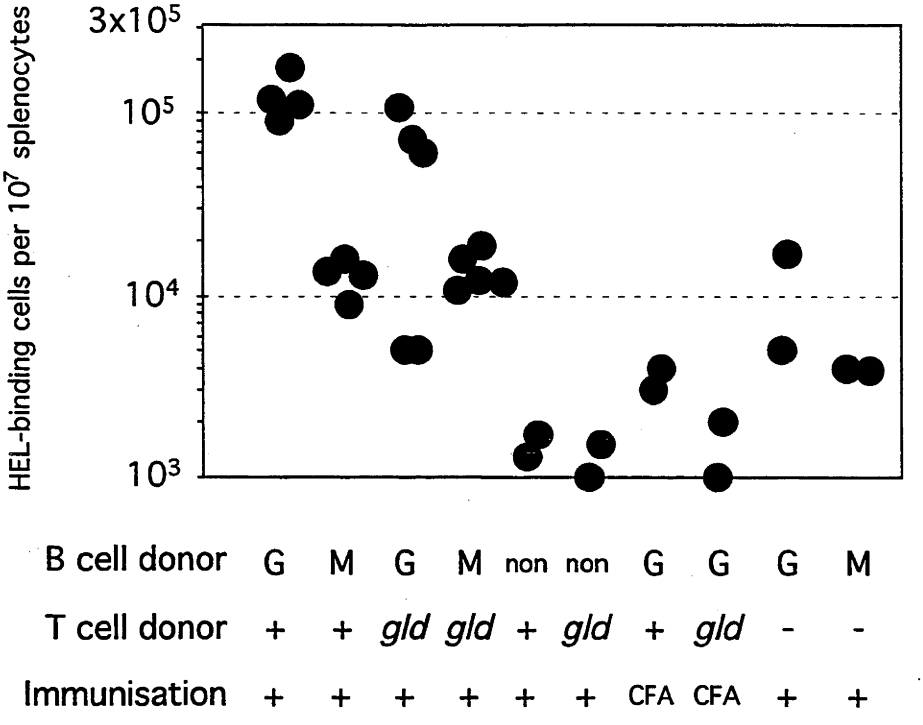
Figure 4-4. Crippling the Fas/FasL pathway in transgenic T cell help does not rescue clonal expansion of IgM transgenic B cells *in vivo*

10^6 IgM:H-2^{kb} or IgG:H-2^{kb} transgenic B cells were transferred along with 10^6 TCR H-2^{kk} or 10^6 TCR:*FasL*^{gld} H-2^{kk} transgenic T cells into B10.BR (H-2^{kk}) recipients immunised at the same time with HEL in CFA or CFA alone.

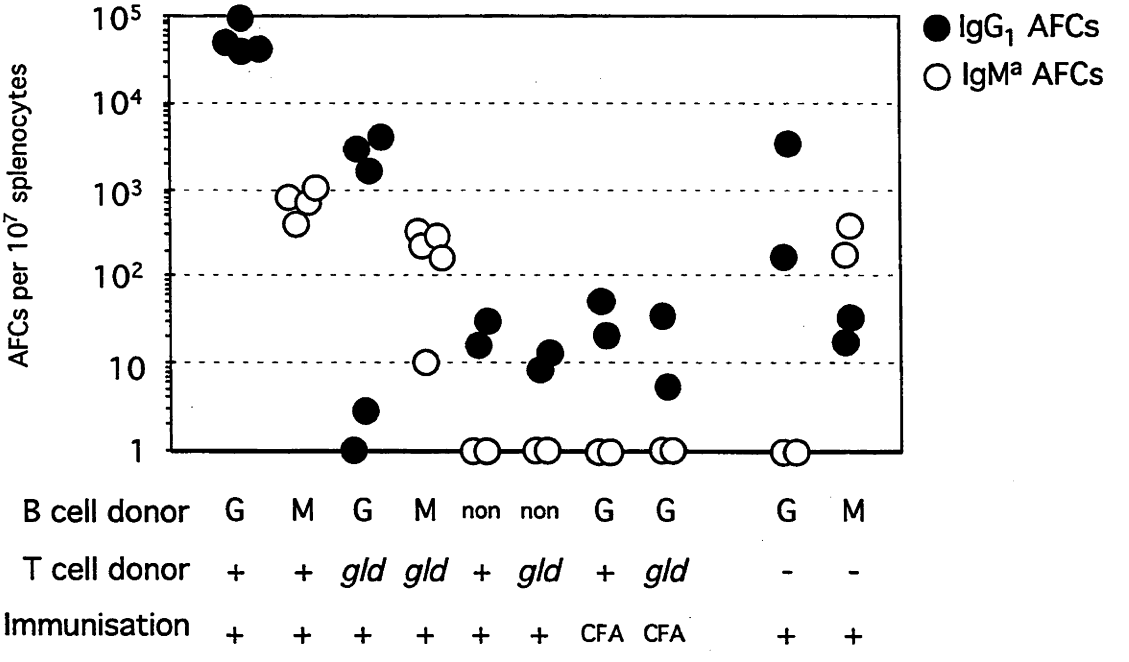
A. Clonal expansion of IgG and IgM transgenic B cells in the presence of TCR or TCR:*FasL*^{gld} transgenic T cells, measured by FACS on day 5 after immunisation and transfer. Data is shown as the number of HEL-binding B cells per 10^7 recipient splenic lymphocytes.

B. ELISPOT measurements of splenic anti-lysozyme IgG_1 and IgM^a AFCs from recipient mouse spleens in (A), measured on day 5 after immunisation and transfer. Data is presented as the number of anti-lysozyme AFCs per 10^7 splenic lymphocytes.

A


HEL-binding cells per 10^7 splenocytes
$3x10^5$
10^5
10^4
10^3
B cell donor G M G M non non G G G M
T cell donor + + gld gld + gld + gld - -
Immunisation + + + + + + CFA CFA + +

B


AFCs per 10^7 splenocytes
10^5
10^4
10^3
10^2
10
1
● IgG_1 AFCs
○ IgM^a AFCs
B cell donor G M G M non non G G G M
T cell donor + + gld gld + gld + gld - -
Immunisation + + + + + + CFA CFA + +

Section 4.5. Do other changes associated with memory play a role in the magnitude of antibody responses?

This study has presented evidence that BCR isotype plays a critical role in primary B cell responses to antigen *in vivo*. To isolate BCR isotype, this has been done using naïve transgenic B cells seeded into a primary response. The role of this phenomenon in memory is more difficult to assess, given the host of other phenotypic changes that accompany the memory state. For example, memory B cells are present in distinct anatomical locations near antigen-draining sites (Liu, 1991; Liu, 1995) and display reduced thresholds of activation, possibly due to their ability to act as APCs (Liu, 1995; Bar-Or, 2001). In addition, memory B cells have been shown to have distinct cell surface phenotypes (Klein, 1998; Tangye, 1998; McHeyzer-Williams, 2000) and in some studies a greatly enhanced lifespan (Schittek, 1990; Maruyama, 2000). This study has shown that the expression of the IgG membrane tail on naïve B cells is sufficient to greatly enhance antigen-specific B cell responses. To assess the role of BCR isotype in memory responses a comparison was attempted between naïve or memory transgenic B cells bearing defined receptor isotypes.

To try to generate Ig transgenic memory cells, IgM and IgM/G transgenic B cells were seeded along with soluble antigen into recipient mice primed with TCR transgenic cells and 100 μg HEL in CFA 6 days previously (Shokat, 1995). It was reasoned that the normal site of memory B cell production is germinal centres rather than extrafollicular sites, so the experimental system that allowed IgM and IgM/G follicular expansion and germinal centre entry was used. The number of antigen-specific B cells was measured on days 4 and 34 after B cell transfer. Recipient mice were maintained for 60 days prior to secondary challenge, in accordance with other studies examining memory B cells (Okomura, 1976; Yefenof, 1985; Hayakawa, 1987; Linton, 1989; Liu, 1991; McHeyzer-Williams, 2000). Recipients were then boosted with 100 μg soluble HEL given i.v. This was done because one of the distinguishing features of the memory antibody response is that antigen need not be given in adjuvant (Liu, 1991; McHeyzer-Williams, 1991). At the same time, separate recipients were immunised with 100 μg HEL in CFA (i.p.) and either 10^6, 10^5 or 10^4 IgM or IgM/G transgenic B cells were transferred along with TCR transgenic cells to simulate a primary immune response and provide a comparison group for the putative "memory" response.

As shown in Figure 4-5, the magnitude of AFC production in response to a secondary boost with soluble antigen on day 65 after priming was much lower than the magnitude of AFC production from 10^6 IgM or IgM/G transgenic B cells seeded into a primary response.

Furthermore, it is impossible to compare the recipients making a primary response with the recipients making a putative memory response, as even on day 34 after B cell transfer into primed mice neither IgM nor IgM/G transgenic B cells could be detected by FACS. The increased numbers of AFCs above background in primed recipients that received IgM/G transgenic cells on day 65 after re-exposure to soluble antigen is suggestive that some antigen-specific cells persisted for this period and were induced to differentiate and secrete antibody on re-exposure to soluble antigen. Without knowing how many cells were present before boosting with soluble antigen it is impossible to know what fold clonal expansion this represents and therefore whether the IgM/G or IgM isotype "memory" cells were more or less responsive to antigen compared to the corresponding "naïve" cells in the primary response.

One problem with this experimental design is the form of antigen used to elicit a "memory" response. Without administration with adjuvant, soluble HEL is cleared very rapidly from the circulation (So, 1999). Nevertheless, soluble antigen elicits a clear secondary response in normal mice in the absence of HEL-specific transgenic B or T cells (Figure 3-1, part B). It is possible that the presence of transgenic T helper cells interfered with the production of memory B cells. The ability of the 3A9 TCR transgenic helper T cells to provide selective signals in the germinal centre is unknown. It would be useful to repeat these experiments in mice that do not receive transgenic T cells. In addition, a more reliable method of detecting antigen-specific memory cells at low frequency must be developed. Marking the transgenic B cells using CD45 allotypic markers in addition to antigen-specificity may allow unequivocal enumeration of frequency prior to the secondary immunisations. This may allow the "clonal burst" of those cells to be accurately measured during the secondary response, to answer the question of whether memory transgenic B cells bearing the IgG membrane tail respond more robustly to antigen.

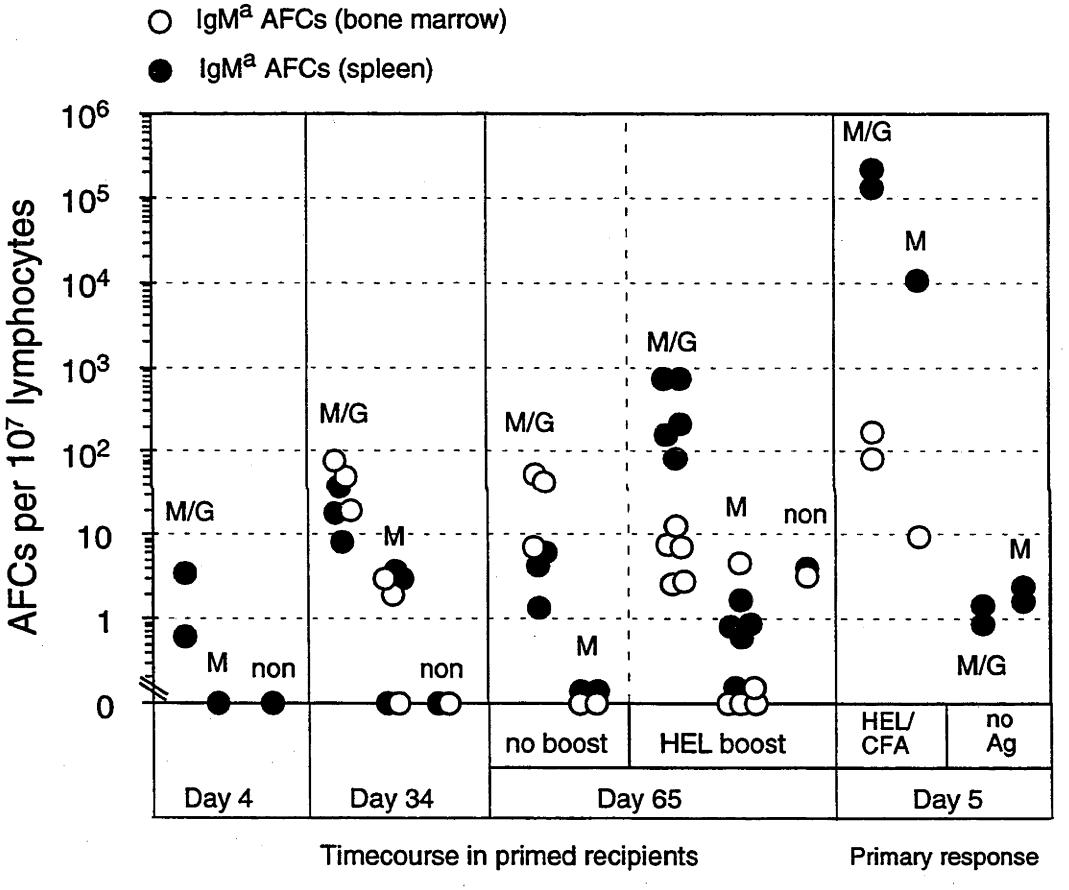
Figure 4-5. Long-term memory B cells are poorly generated from naïve transgenic B cells in short-term primed recipients

Recipient (B10.BRxC57BL/6)F_1 mice were primed with 10^6 TCR transgenic T cells and antigen in adjuvant 6 days before the transfer of 10^6 of the indicated type of transgenic B cell boosting soluble antigen.

On days 4 and 34, recipient spleen and bone marrow in primed mice was analysed for lysozyme-specific IgM^a AFCs. No HEL-specific B cells could be detected in the spleen at either timepoint by FACS.

On day 60, recipients were boosted with soluble antigen (100 μg HEL in PBS, i.v.) and analysed by ELISPOT on day 65. In parallel on day 60, unprimed recipient mice were immunised with HEL in CFA and received 10^6 naïve IgM or IgM/G transgenic B cells with 10^6 naïve TCR transgenic T cells, as a comparison group. Bone marrow and spleen samples from these mice were also analysed on day 65 by ELISPOT.

The graph shows the levels of IgM^a anti-lysozyme AFCs in the spleen and bone marrow for the various experimental groups. The labels M/G, M and non denote IgM/G transgenic, IgM transgenic or non-transgenic donor lymphocytes. Data is shown as the number of anti-lysozyme IgM^a AFCs per 10^7 recipient splenic lymphocytes.


IgM[a] AFCs (bone marrow)
IgM[a] AFCs (spleen)
AFCs per 10[7] lymphocytes
10[6]
10[5]
10[4]
10[3]
10[2]
10
1
0
M/G
M
non
M/G
M
non
M/G
M
non
M/G
M
M/G
M
M/G
no boost
HEL boost
HEL/ CFA
no Ag
Day 4
Day 34
Day 65
Day 5
Timecourse in primed recipients
Primary response

Section 4.6. Chapter summary

This chapter extends the evidence presented in Chapter 3 suggesting that differences in IgM, IgG and IgM/G transgenic B cell clonal expansion and effector cell production are quantitative rather than qualitative. The previous chapter showed an equivalent rate of cell activation and cell division between IgM, IgG and IgM/G transgenic B cells but a large difference in net clonal expansion on day 5 during a primary anti-lysozyme response.

This chapter has presented a more detailed analysis of events between days 3 and 5 after transfer and immunisation. It has shown that the differences between the transgenic B cells expressing or lacking the IgG membrane tail manifest during an immune response at the time when extrafollicular foci disperse to generate syndecan-positive effector cells in the bridging channels and red pulp. IgM transgenic cells progress through a similar number of cell division cycles between day 3 and day 5 compared to IgM/G transgenic B cells, however produce far fewer effector cells. The hypothesis that the IgM cells are dying during clonal expansion in the spleen is supported by the lack of AFC production in the bone marrow. Finally, transgenic helper T cell–induced Fas/FasL apoptosis is not responsible for the poor expansion of IgM transgenic cells, as crippling this pathway does not rescue IgM clonal expansion and effector production. Apoptotic cell death is not ruled out by this finding, however, as endogenous recipient T cells and DCs could still express FasL.

The poor net clonal burst of IgM transgenic B cells is not the result of a differential preference for germinal centre versus extrafollicular clonal expansion. IgM and IgM/G transgenic cells can participate in both pathways, a decision possibly mediated by the type and location of transgenic T cell help during the primary or pseudo-primary immune responses described here.

Finally, an attempt was made to test the relative importance of BCR isotype versus other phenotypic and functional changes in the qualities of the memory response. This is an important extension of the work presented in Chapter 3, as while the use of naïve Ig transgenic B cells is critical in isolating BCR isotype from the other phenotypic changes that underlie antigen experience, that strategy gives no indication of the relative importance of BCR isotype versus these other changes during a recall response. To address this, it was reasoned that comparisons of transgenic memory B cells expressing IgG or IgM isotype BCRs to naïve transgenic B cells bearing the same isotypes would show whether other factors associated with the memory state alter responses to antigen. To generate memory transgenic B cells, naïve transgenic B cells were seeded into short-term primed recipient

mice. This approach was not successful, as technical difficulties such as poor memory cell formation and persistence did not allow any conclusions to be made about the relative reactivity of memory or naïve transgenic B cells.

Section 4.7. Future Work

In the future it will be important to directly test some of the predictions in this chapter, namely that IgM transgenic B cells fail to make a strong clonal expansion response because they undergo apoptosis, due to interaction with a highly cross-linking form of immune-complexed HEL held by dendritic cells.

Histologically it may be possible to demonstrate a co-localisation of $CD11c^{hi}$ "PDCs" and Ig transgenic B cells in the extrafollicular foci seen on days 3 and 4 after immunisation during the primary response. This may help determine whether these dendritic cells are involved in the pattern of cell loss observed in IgM transgenic B cells.

In addition, crossing the IgM, IgG and IgM/G transgenic lines with mice overexpressing anti-apoptotic proteins such as Bcl-2 will show whether apoptosis is the mechanism of IgM cell loss. If Bcl-2 overexpression restores IgM clonal expansion to similar levels compared to IgG and IgM/G transgenic B cells, this would provide good evidence that apoptosis is responsible for the loss of IgM transgenic B cells normally in this system.

It may be possible to determine the role of immune complex in IgM transgenic cell loss by performing these transfer experiments in RAG-deficient mice that are unable to produce endogenous, lower affinity multivalent IgM. If IgM transgenic cell clonal expansion is rescued under those conditions it would then be possible to introduce immune complex and determine whether immune complex can induce cell loss.

Connected to this issue is the observation that transgenic B cells on day 4 of a primary response in the presence of transgenic T cell help are undetectable on the basis of BCR staining. We would hypothesise that this is due to BCR downmodulation after contact with mulit-valent HEL presented as immune complex. Transfer experiments using CD45 allotypic markers would allow transgenic B cells to be tracked at this timepoint without relying on surface BCR expression. This would allow the accurate enumeration of transgenic B cells during the later phase of clonal expansion.

In order to complement the current study, transfer experiments will be performed using B cells from anti-HEL knockin mice (a kind gift from Dr. J. G. Cyster, UCSF). This

will help to show the normal magnitude and locations of primary IgM and IgG responses in B cells that can switch isotype. This will be important for determining whether the primary responses by the Ig transgenic IgM and IgG B cells used in this study deviate from a situation where isotype switching is normal.

Also, striking differences in the location of clonal expansion of Ig transgenic B cells was noticed between a primary response with naïve T cells and a pseudo-primary response with primed T cells. It would be interesting to use CD45 allotypic markers and anti-clonotype antibodies to track the expansion of 3A9 TCR transgenic T cells over the course of these experiments. To extend this, the cell surface phenotype of the transgenic T cells could be assessed. For example, the expression of the chemokine receptor CXCR5 seems to be a useful marker to distinguish $CD4^+$ T cells that are capable of entering B cell follicles (Ansel, 1999; Breitfeld, 2000; Schaerli, 2000; Kim, 2001). Staining for this, in conjunction with immunohistochemistry to localise the transgenic T cells would provide a powerful way of addressing the issue of the type and location of T cell help during these responses.

Perhaps the most critical future work to extend the experiments presented in this chapter is to determine the relative importance of BCR isotype versus other antigen-induced changes in memory B cells that determine the magnitude of the memory response. It will be important to repeat similar experiments to that described in Section 4.5 in an attempt to generate memory B cells that have defined BCR isotypes. As well as enumerating the memory cells at various timepoints, it will be interesting to see where they localise, what surface phenotype they adopt and how they behave when sorted populations bearing different isotypes are transferred into immunised recipient mice.

Chapter 5. Marginal zone and follicular B cell subsets vary in different anti-HEL transgenic lines

Section 5.1. Introduction

The marginal zone and follicular B cell subsets represent phenotypic and geographically distinct compartments of the spleen. In this chapter these subsets were examined in a range of anti-HEL transgenic mouse lines bearing a variety of different BCR isotypes.

Marginal zone or follicular enrichment of particular B cell clones has been demonstrated in several transgenic systems (Martin, 2000b), including marginal zone enrichment in the Hc transgenic 81x and M167lines and follicular enrichment in the anti-HEL MD2 line. It is hypothesised that the enrichment phenotypes in the marginal zone depend on some quality of BCR signalling, as they are absent in Btk^{xid} animals (Martin, 2000b).

Previous observations of the B cell subsets in mice carrying the IgG membrane tail showed an accumulation of $CD21^{hi}$ HEL-binding B cells in those lines (Pogue, 1996; Pogue, 2000), which was suggestive of a marginal zone phenotype. This was an interesting observation that may impact on the adoptive transfer system discussed in the previous chapters. Marginal zone cells have been associated with rapid effector cell production during memory (Liu, 1991) and T-independent type II responses (Martin, 2000a), so differences in HEL-binding marginal zone B cell fractions between IgG, IgM/G and IgM transgenic lines may provide a mechanism for their differential reactivity to antigen on adoptive transfer.

This chapter will present evidence that there are striking differences in the proportion of HEL-binding cells present in the marginal zone subsets of a range of anti-HEL transgenic lines expressing a variety of BCR isotypes. While individual anti-HEL BCR isotypes seem more or less able to support marginal zone entry and accumulation, the analysis is complicated by a correlation between the degree of marginal zone entry and the proportion of competing, endogenous B cells present in each transgenic line.

Section 5.2. Peripheral B cell subsets in IgG, IgM/G and IgM transgenic lines

A consistent finding in this study and previous work by Pogue and Goodnow (Pogue, 1996; Pogue, 2000) was a decreased proportion of antigen-binding transgenic B cells in the periphery of mice expressing IgG or chimeric IgM/G BCR isotypes. This selection away

from HEL-binding was shown to correlate with transgene copy number and may reflect stronger signalling *via* the IgG isotype when it is expressed early in B cell development, leading to some form of receptor editing (Pogue, 2000). However, as well as dictating the proportion of peripheral HEL-binding B cells, expression of the IgG membrane tail produced an unusual pattern of CD21 expression on mature, peripheral HEL-binding B cells when compared to IgM transgenic B cells.

Figure 5-1 shows representative FACS plots from IgG (GG4), IgM/G (MG2), IgM (MM4) and non-transgenic control spleen cells on the (B10.BRxC57BL/6)F_1 background. Note that the proportion of HEL-binding lymphocytes is reduced in IgG (~10 %) and IgM/G (~18 %) transgenic mice compared to IgM transgenic mice (~28 %) (Figure 5-1, part A). In addition, the CD21 profiles of the HEL-binding cells show a predominant $CD21^{hi}$ and smaller $CD21^{med}$ population in both IgG and IgM/G transgenic lines compared to the main $CD21^{med}$ population in the IgM transgenic line or non-transgenic control (Figure 5-1, part B). Interestingly, the proportion of transgenic cells in the $CD21^{hi}$ subset is even more pronounced in the IgM/G transgenic line compared to the IgG transgenic line (52 % IgM/G versus 40 % IgG), and this is a consistent finding (see Table 5-1).

Section 5.3. Marginal zone and follicular B cell analysis in IgG, IgM/G and IgM transgenic lines

High expression of CD21 has been associated with marginal zone and transitional type 2 B cells, and in conjunction with CD23 allows the marginal zone ($CD21^{hi}$, $CD23^-$) and follicular ($CD21^{med}$, $CD23^+$) subsets to be distinguished (Loder, 1999). To investigate whether the enlarged $CD21^{hi}$ population in the IgG and IgM/G transgenic lines was due to changes in the marginal zone B cell compartment, spleen cells were stained for CD21, CD23 and HEL to distinguish transgenic and non-transgenic marginal zone and follicular subsets (Figure 5-2).

Figure 5-2, part A is gated on lymphocytes to show the marginal zone ($CD21^{hi}$, $CD23^-$), follicular ($CD21^{med}$, $CD23^+$) and other lymphocyte ($CD21^{lo/-}$, $CD23^-$) subsets from representative IgG, IgM/G, IgM and non-transgenic mouse spleens. In this case, the marginal zone compartment in IgG (~16 %) and IgM/G (~20 %) transgenic mice was expanded relative to the marginal zone in IgM (~8 %) transgenic mice or non-transgenic (~ 8 %) controls. Figure 5-2, part B shows the relative proportion of HEL-binding and non-transgenic B cells in both the marginal zone (M) gate and follicular (F) gate. The most striking finding was the partitioning of HEL-binding cells between the MZ and FO compartments in the IgM transgenic line. Over 90 % of the follicular subset in those mice

Figure 5-1. Peripheral B cells in IgG, IgM/G and IgM transgenic lines vary in CD21 expression profile and in the fraction of B cells that bind HEL

FACS plots show representative IgM, IgM/G, IgG transgenic and non-transgenic spleen cells. The IgG Tg, IgM Tg and non-Tg samples were stained and analysed at the same time. The IgM/G Tg sample was stained and analysed in a separate experiment. The first set of plots show the lymphocyte gates set on forward and side scatter. The numbers refer to the fraction of cells within the gate.

A. HEL-binding versus CD21 expression on gated lymphocytes, showing the variable proportion of HEL-binding B cells in each line (also see Table 5-1). Numbers refer to the fraction of lymphocytes within the HEL-binding gate.

B. Histograms showing CD21 expression, gated on the HEL-binding lymphocytes from (A) for IgG, IgM and IgM/G transgenic samples, or gated on $CD21^{+}$ lymphocytes in the non-transgenic control. The histograms highlight the variation in $CD21^{lo}$, $CD21^{med}$ and $CD21^{hi}$ subsets between the transgenic lines. Numbers refer to the fraction of cells in the $CD21^{lo}$, $CD21^{med}$ and $CD21^{hi}$ regions.

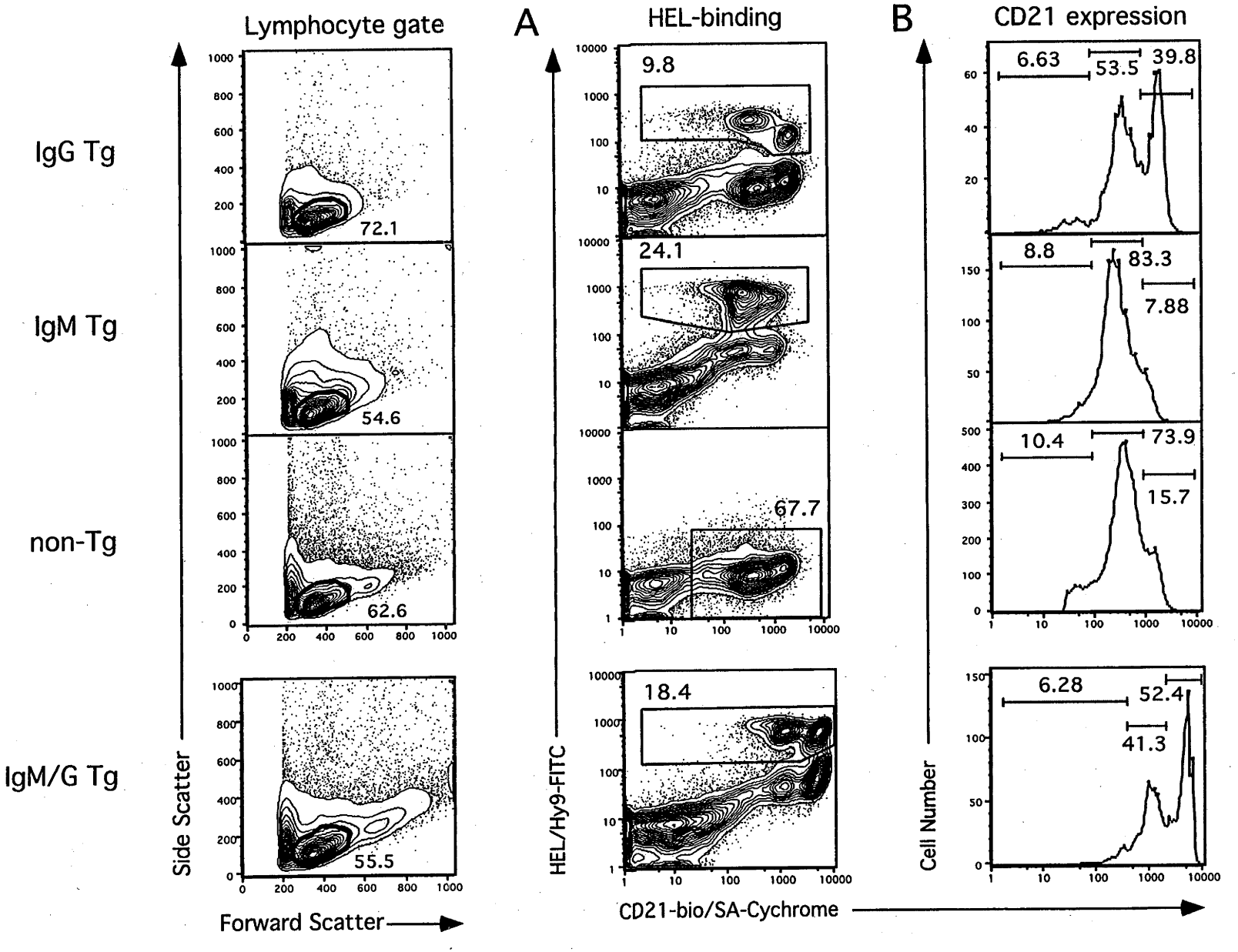

Lymphocyte gate
A
HEL-binding
B
CD21 expression
IgG Tg
IgM Tg
non-Tg
IgM/G Tg
72.1
54.6
62.6
55.5
9.8
24.1
67.7
18.4
6.63
53.5
39.8
8.8
83.3
7.88
10.4
73.9
15.7
6.28
52.4
41.3
Side Scatter
Forward Scatter
HEL/Hy9-FITC
CD21-bio/SA-Cychrome
Cell Number

Figure 5-2. IgG, IgM/G and IgM transgenic spleens show different recruitment of peripheral B cells into the splenic marginal zone and follicular subsets by FACS

A. FACS plots gated on splenic lymphocytes (see Figure 5-1), showing IgG, IgM/G, IgM transgenic and non-transgenic marginal zone ($CD21^{hi}$ $CD23^{-}$), follicular ($CD21^{med}$ $CD23^{+}$) and other lymphocyte ($CD21^{lo}$ $CD23^{-}$) subsets. Numbers indicate the fraction of lymphocytes falling within each gate.

B. Histograms showing HEL-binding BCR expression levels gated on marginal zone (M) or follicular (F) subsets from (A). Two regions are shown on each histogram, HEL-binding and non-binding lymphocytes. The numbers refer to the fraction of gated cells falling in each region.

C. Histograms showing forward scatter versus cell number for each sample, gated on the marginal zone (M) or follicular (F) subsets from (A).

D. Gated lymphocytes were first sub-divided into HEL^{+} and HEL^{-} subsets as depicted by regions on HEL-binding histograms, and CD21 versus CD23 contour plots produced to determine the marginal zone and follicular fractions in each subset. Numbers on the contour plots refer to the fraction of HEL-binding or non-binding lymphocytes falling within the marginal zone ($CD21^{hi}$ $CD23^{-}$), follicular ($CD21^{med}$ $CD23^{+}$) or other lymphocyte ($CD21^{lo}$ $CD23^{-}$) gates.

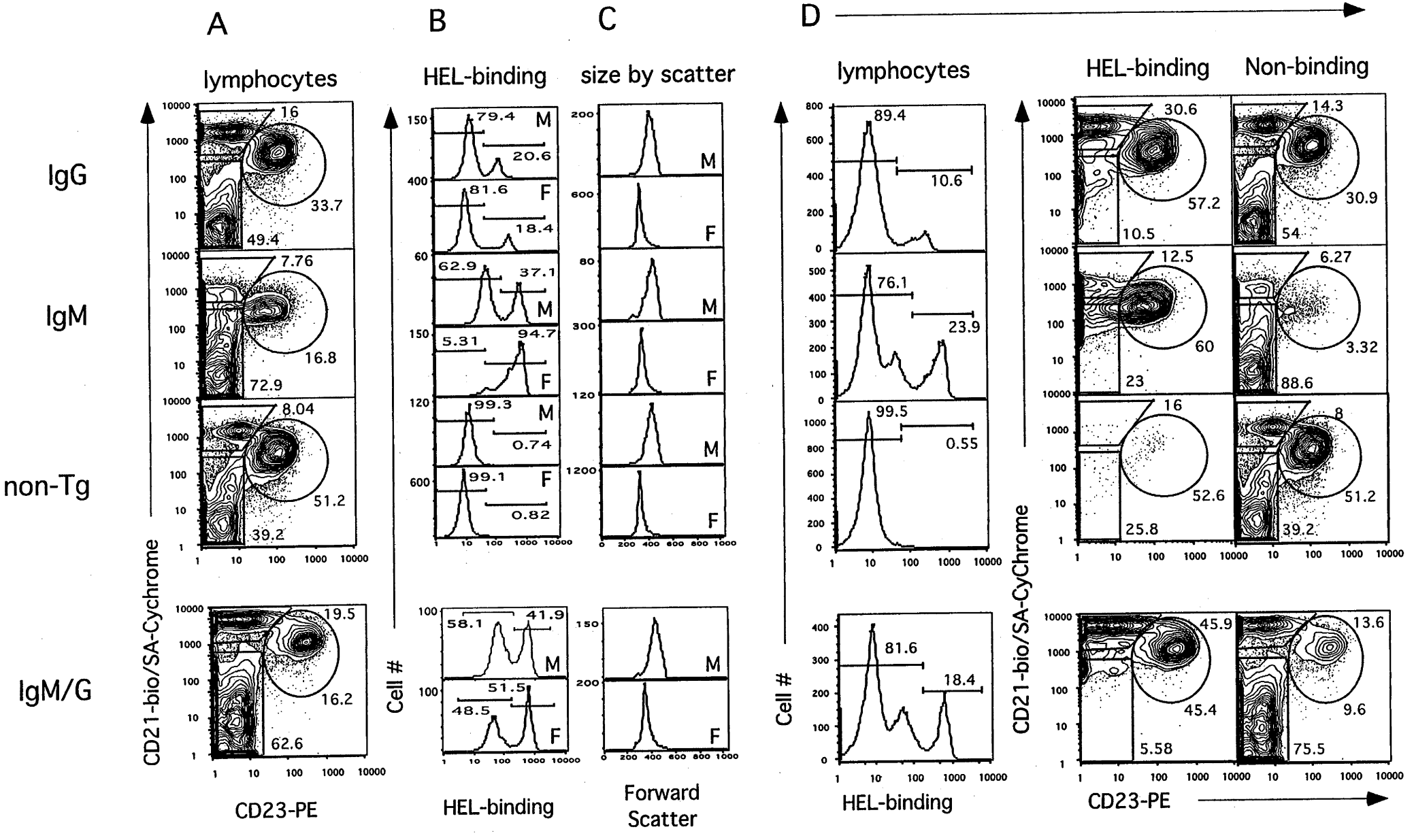

A
B
C
D
lymphocytes
HEL-binding
size by scatter
lymphocytes
HEL-binding
Non-binding
IgG
IgM
non-Tg
IgM/G
16
33.7
49.4
7.76
16.8
72.9
8.04
51.2
39.2
19.5
16.2
62.6
79.4
M
20.6
81.6
F
18.4
62.9
37.1
M
5.31
94.7
F
99.3
M
0.74
99.1
F
0.82
58.1
41.9
M
51.5
48.5
F
M
F
M
F
M
F
89.4
10.6
76.1
23.9
99.5
0.55
81.6
18.4
30.6
57.2
10.5
14.3
30.9
54
12.5
60
23
6.27
3.32
88.6
16
52.6
25.8
8
51.2
39.2
45.9
45.4
5.58
13.6
9.6
75.5
CD21-bio/SA-Cychrome
Cell #
CD23-PE
HEL-binding
Forward Scatter
HEL-binding
CD21-bio/SA-CyChrome
CD23-PE

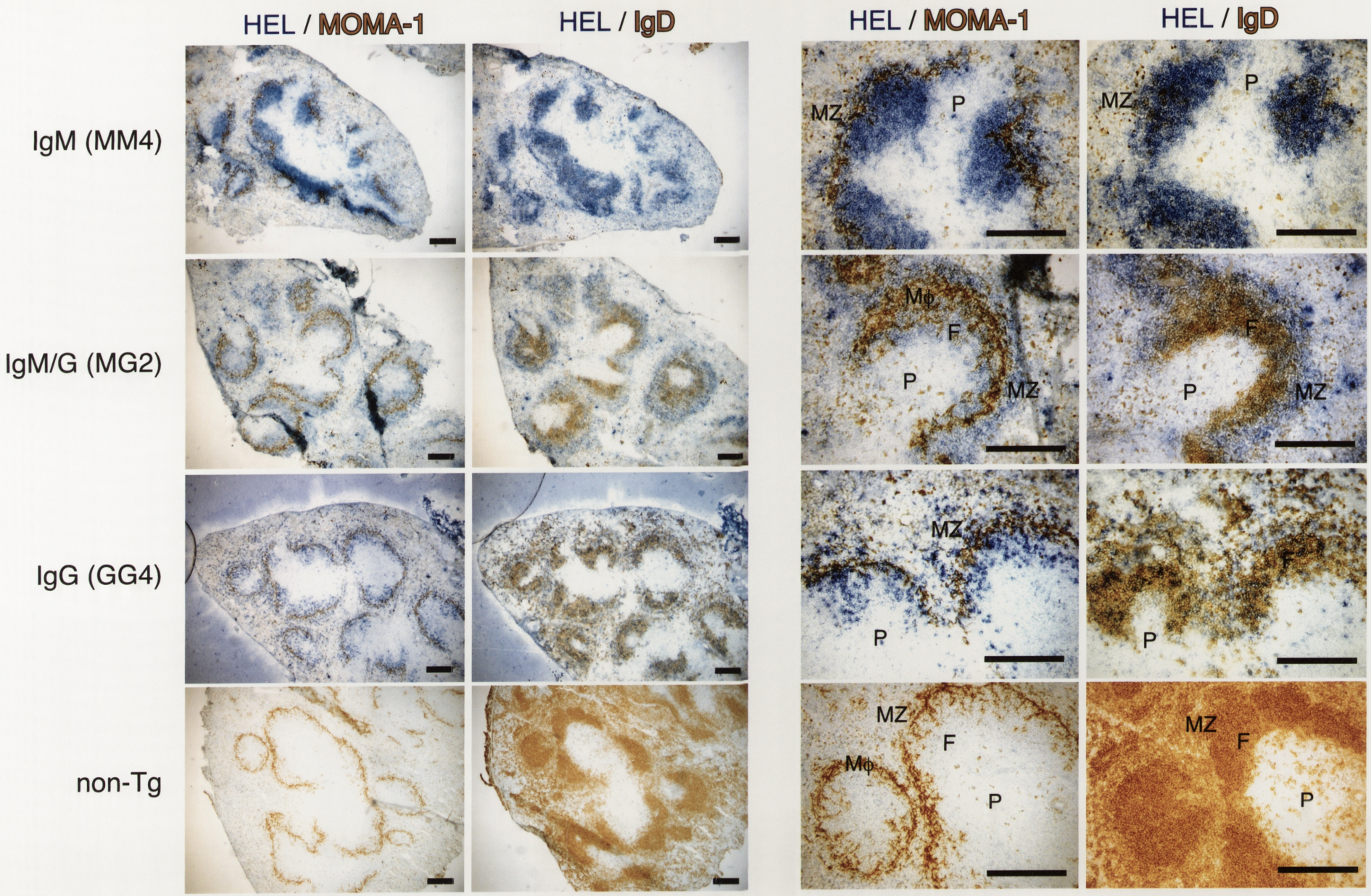
Figure 5-3. Spleen histology showing the location of HEL-binding cells in IgG, IgM/G and IgM transgenic mice

Serial 5 μm frozen spleen sections from IgG, IgM/G, IgM transgenic and non-transgenic mice were stained for the presence of HEL-binding cells (blue), and for either IgD or MOMA-1 in brown, as indicated above each column.

The IgD stain shows the B cell follicles, while the MOMA-1 stain shows the marginal zone metalophilic macrophages that line the follicular side of the marginal sinus.

The first two columns show a lower power view of the spleen, and the second two columns are high-powered fields from representative areas of the same samples. Bars represent 200 μm.

High powered fields are annotated, showing the location of the splenic marginal zone (MZ), B cell follicles (F), PALS (P) and metalophilic macrophages (Mϕ).


HEL / MOMA-1
HEL / IgD
HEL / MOMA-1
HEL / IgD
IgM (MM4)
IgM/G (MG2)
IgG (GG4)
non-Tg
MZ
P
Mϕ
F

were HEL-binding cells, while only 37 % of the marginal zone subset bound HEL. Figure 5-2, part C shows that the marginal zone compartment (M) in all lines contains larger cells on the basis of forward scatter compared to the follicular compartment (F), a characteristic phenotype of MZ B cells noted by others (Oliver, 1997a). Figure 5-2, part D shows the relative marginal zone and follicular partitioning of HEL-binding (HEL^+) and other (HEL^-) lymphocytes in the various lines. HEL-binding lymphocytes were equivalently partitioned between MZ and FO subsets in IgM/G transgenic mice, are present in a 2:1 FO: MZ ratio in IgG transgenic mice and are predominantly follicular (5:1 FO: MZ) in IgM transgenic mice.

The results from multiple animals from a range of strains is also consistent with wide variation in the fraction of HEL-binding cells that have a marginal zone or follicular phenotype, ranging from ~68 % marginal zone in the MM4xDD6 line to ~10 % marginal zone in the MM4 line (Table 5-1).

Histological examination of IgG, IgM/G and IgM transgenic lines also supported the FACS data showing differences in HEL-binding marginal zone and follicular compartments (Figure 5-3). In IgG transgenic spleen sections, HEL-binding B cells were visible throughout the marginal zone and follicular areas. In IgM/G transgenic spleen sections, HEL-binding B cells were clearly positioned towards the outer edge of the B cell follicle, with IgD-expressing endogenous B cells compressed towards the PALS. Using MOMA-1 staining to illuminate the marginal zone metallophilic macrophages, many IgM/G HEL-binding B cells surrounded the marginal sinus on the red pulp side, consistent with the marginal zone cell surface phenotype of a larger fraction of HEL-binding B cells in this line. By contrast, IgM transgenic B cells showed a preference for the follicular area of the spleen, as sections co-stained with MOMA-1 and HEL-binding showed the majority of HEL-binding B cells are on the follicular side of the metalophilic macrophages. Again, this is consistent with the FACS data showing that most HEL-binding B cells in this line have a follicular phenotype.

Section 5.4. Marginal zone and follicular subset differences are retained and augmented in $RAG1^{-/-}$ mice

The histological and FACS data clearly illustrate striking differences in the partitioning of HEL-binding and non-binding B cells in the marginal zone and follicular compartments of different Ig transgenic mice. It was important to find out whether the

increased entry of HEL-binding B cells into the marginal zone compartment in IgG and IgM/G transgenic cells was due to the presence of the competing "non-transgenic" B cells in the follicles or marginal zone, or due to some T-dependent antigen-driven process. To answer this, IgG (GG4), IgM/G (MG2) and IgM (MM4) transgenic lines were crossed to the $RAG1^{-/-}$ background. $RAG1^{-/-}$ IgG, IgM/G and IgM transgenic mice produce similar proportions of mature peripheral HEL-binding B cells, as shown in Figure 5-4, panel A and in Table 5-1. Only ~10 % of IgM:$RAG1^{-/-}$ HEL-binding B cells have a marginal zone phenotype, while a much larger proportion of HEL-binding B cells in IgG:$RAG1^{-/-}$ (~64 %) and IgM/G:$RAG1^{-/-}$ (~43 %) transgenic mice show a marginal zone phenotype (Figure 5-4 and Table 5-1).

The relative accumulation of HEL-binding B cells into the marginal zone in the IgM transgenic line is not affected by the removal of competing B cells or T cells, as the majority of HEL-binding B cells in the IgM:$RAG1^{-/-}$ mice retain a follicular phenotype. This is not the case for the IgG and IgM/G transgenic lines. On the $RAG1^{+/+}$ background, a greater proportion of IgM/G HEL-binding cells were in the marginal zone subset compared to the IgG transgenic line (IgG:$RAG1^{+/+}$, 30.9 % ± 5.8, n = 6; IgM/G:$RAG1^{+/+}$, 48.6 % ± 4.9, n = 13, see Table 5-1). On the $RAG1^{-/-}$ background these differences are no longer apparent, and a similar proportion of HEL-binding B cells accumulate in the marginal zone subset in each line (IgG:$RAG1^{-/-}$, 57.7 % ± 8.5, n = 11; IgM/G:$RAG1^{-/-}$, 58.0 % ± 4.1, n = 5, see Table 5-1).

Why do the differences in the marginal zone subset between IgG and IgM/G lines disappear on the $RAG1^{-/-}$ background? The key may be the removal of clonal competition between HEL-binding and other B cell specificities for marginal zone entry. HEL-binding IgG B cells form a smaller fraction of total peripheral B cells compared to IgM/G HEL-binding B cells (see Figure 5-1 and Table 5-1). This implies a greater degree of competition for marginal zone entry between HEL-binding and non-binding B cells in the IgG:$RAG1^{+/+}$ line compared to the IgM/G:$RAG1^{+/+}$ line. When competing B cells are removed by crossing to $RAG1^{-/-}$, the marginal zone bias of the IgG membrane tail is separated from competition effects, and both IgG and IgM/G transgenic cells fill the marginal zone to the same extent.

There is already data suggesting that inter-clonal competition affects B cell entry into the marginal zone. In the HEL system, most B cells in the Hc+Lc MD4 line are HEL-binding and form a well-developed marginal zone (Mason, 1992). In the MD2 Hc-only transgenic line, a much smaller proportion of B cells are HEL-binders. Under these conditions, very few HEL-binding cells are found in the marginal zone, presumably due to

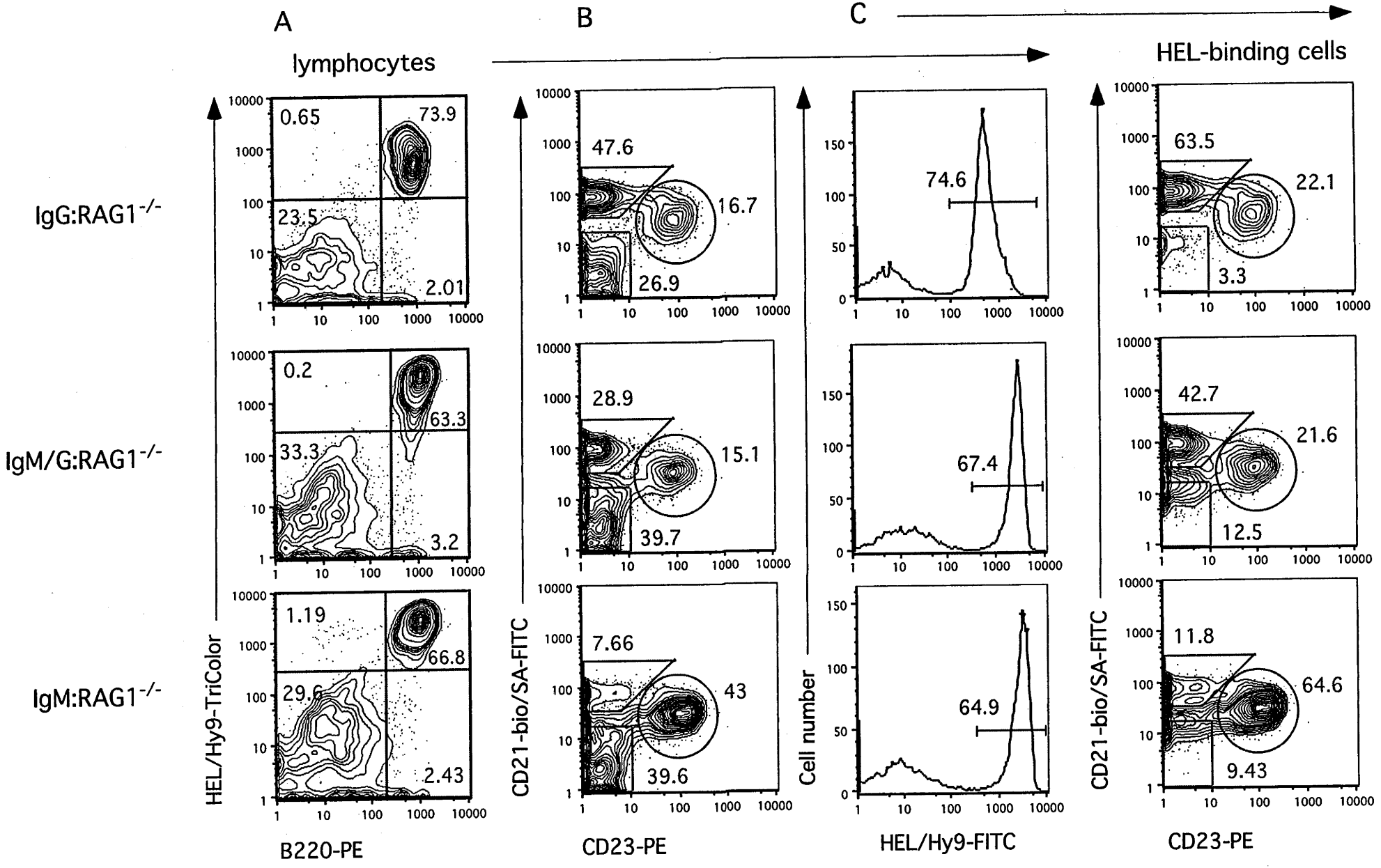
increased competition from non-HEL-binding B cells bearing other specificities (Martin, 2000b). The same phenomenon has also been observed in the 81x transgenic system. In 50:50 competition bone marrow chimeras, only a small fraction (< 3 %) of the $CD21^{hi}$ marginal zone subset was composed of Tg-μ positive B cells, whereas in the normal 81x Tg mice the $CD21^{hi}$ compartment was filled with B cells expressing the Tg-μ heavy chain (Martin, 1997).

Figure 5-4. Increased entry into the marginal zone compartment by IgG and IgM/G transgenic B cells still occurs on the RAG1$^{-/-}$ background

A. Contour plots of IgG: RAG1$^{-/-}$, IgM/G: RAG1$^{-/-}$ and IgM: RAG1$^{-/-}$ transgenic spleen cells gated on lymphocytes by forward and side scatter, showing HEL-binding versus B220. Numbers indicate the fraction of lymphocytes within each quadrant. Note the similar proportion of HEL-binding B220^{+} lymphocytes in each line.

B. Contour plot showing marginal zone (CD21^{hi} CD23^{-}) versus follicular (CD21^{med} CD23^{+}) subsets in the RAG1$^{-/-}$ transgenic spleen samples, gated first on small lymphocytes. Numbers indicate the fraction of lymphocytes within each gate.

C. Contour plot showing marginal zone and follicular HEL-binding spleen subsets, gated first on HEL-binding lymphocytes using the indicated histogram gates. Numbers indicate the fraction of HEL-binding lymphocytes within each gate.


A
B
C
lymphocytes
HEL-binding cells
IgG:RAG1-/-
IgM/G:RAG1-/-
IgM:RAG1-/-
0.65
73.9
23.5
2.01
0.2
63.3
33.3
3.2
1.19
66.8
29.6
2.43
HEL/Hy9-TriColor
B220-PE
47.6
16.7
26.9
28.9
15.1
39.7
7.66
43
39.6
CD21-bio/SA-FITC
CD23-PE
74.6
67.4
64.9
Cell number
HEL/Hy9-FITC
63.5
22.1
3.3
42.7
21.6
12.5
11.8
64.6
9.43
CD21-bio/SA-FITC
CD23-PE

Section 5.5. Marginal zone and follicular subset differences are present in other HEL transgenic lines

The accumulation of HEL-binding cells in the marginal zone compartment was not restricted to lines expressing the IgG membrane tail. In fact, the other transgenic lines tested showed a wide variety of HEL-binding marginal zone and follicular subsets (Table 5-1). It is important to note that the assignment of "marginal zone" and "follicular" phenotypes to these subsets has been performed by FACS, in the future histological examination will be necessary to confirm these data.

Two factors may combine to produce the observed patterns of marginal zone and follicular B cells in this system. Firstly, marginal zone recruitment could reflect some quality of "basal" BCR signalling through the various combinations of anti-HEL Ig isotypes. If this is correct, some isotype-specific differences must play a role in driving accumulation in one subset or another, as the BCRs expressed in these lines share identical variable regions, so would interact equivalently with any selecting ligands. The possibility of isotype-specific signalling leading to marginal zone enrichment is most clearly illustrated in the increased proportion of HEL-binding B cells in the marginal zone of IgG:RAG1$^{-/-}$ and IgM/G:RAG1$^{-/-}$ transgenic mice compared to IgM:RAG1$^{-/-}$ mice. Secondly, the degree of marginal zone recruitment of HEL-binding B cells may also be dependent on the level of competition provided by other B cells in the spleen that do not express the transgenic receptor. This possibility is illustrated by the unequal fraction of marginal zone HEL-binding B cells in IgG and IgM/G animals in the presence of variable numbers of endogenous B cells on the RAG1$^{+/+}$ background.

These factors are difficult to separate, unless competition can be completely removed, as in the IgM, IgG and IgM/G lines crossed to the RAG1$^{-/-}$ background. However, there are several difficulties in analysing all of the transgenic lines in this way. Firstly, RAG1$^{-/-}$ mice lack an adaptive immune system, with no natural antibody and disturbed gut immunity. This complicates the simple interpretation of the removal of B cell competition by introducing several new variables. Secondly, it would be very time consuming to cross all of the transgenic lines to RAG1$^{-/-}$.

Alternatively, the effect of competition from non-transgenic B cells on marginal zone entry by HEL-binding cells can be examined by comparing the proportion of transgenic marginal zone cells with the proportion of splenic lymphocytes that bind HEL across the

various lines. This compares the degree of marginal zone enrichment by HEL-binding B cells with the degree of clonal competition provided by non-HEL-binding B cells bearing endogenous specificities. Figure 5-5 shows the result of this analysis. In general, as the proportion of "competing" non-transgenic B cells increases, the proportion of HEL-binding cells that have a marginal zone phenotype decreases (trend indicated by the filled circles). There are, nevertheless, some clear exceptions to this trend (open circles). Most strikingly, the IgM-only MM4 transgenic line has comparatively few HEL-binding marginal zone cells even on the RAG1$^{-/-}$ background in the complete absence of competing B cells. By contrast, the IgM/G chimeric line MG8 has few HEL-binding B cells and many competing non-transgenic B cells, yet a large proportion of the HEL-binding B cells have a marginal zone phenotype.

Table 5-1. Cell numbers and the fractions of HEL-binding B cells in marginal zone and follicular subsets for each transgenic line

Each of the transgenic lines used in this study is listed. The numbers in parentheses refer to the number of individual animals tested for each criterion. The range indicated is ± 1 standard error and is shown only for measurements with >2 repetitions.

The number of splenic lymphocytes denotes cells counted by hemocytometer as described in section 2.6 (b) of the materials and methods. It is expressed as the number of lymphocytes per spleen.

The other criteria have been determined by FACS stains, as described in the legends of Figure 5-1, Figure 5-2 and Figure 5-4.

The % of $B220^+$ HEL^+ lymphocytes denotes the fraction of lymphocytes gated by scatter that stain for HEL-binding and B220.

The % of marginal zone and follicular lymphocytes denotes the fraction of total lymphocytes gated by scatter that have either a marginal zone ($CD21^{hi}$ $CD23^-$) or follicular ($CD21^{med}$ $CD23^+$) phenotype.

The % of HEL^+ marginal zone or follicular lymphocytes denotes the fraction of HEL-binding lymphocytes that have either a marginal zone ($CD21^{hi}$ $CD23^-$) or follicular ($CD21^{med}$ $CD23^+$) phenotypes.

Transgenic line	Splenic lymphocytes ($x10^7$)	% $B220^+$ HEL^+ lymphocytes	% Marginal zone lymphocytes	% Follicular lymphocytes	% HEL^+ MZ lymphocytes	% HEL^+ FO lymphocytes
GG4	8.4 ± 1.6 (9)	11.1 ± 1.4 (9)	14.1 ± 2.3 (6)	29.2 ± 2.7 (6)	30.9 ± 5.8 (6)	61.0 ± 7.7 (6)
MG2	6.2 ± 1.2 (14)	17.5 ± 3.8 (14)	23.2 ± 2.6 (13)	13.2 ± 3.1 (13)	48.6 ± 4.9 (13)	37.3 ± 8.6 (13)
MG6	6.3 ± 1.6 (5)	8.1 ± 2.6 (5)	16.8 ± 2.3 (3)	29.8 ± 1.2 (3)	19.0 ± 3.6 (3)	34.4 ± 2.3 (3)
MG8	4.7 ± 1.3 (7)	5.9 ± 2.1 (7)	24.2 ± 2.2 (4)	16.7 ± 1.6 (4)	58.8 ± 3.7 (4)	19.7 ± 1.5 (4)
MM4	5.9 ± 1.3 (15)	28.6 ± 3.4 (15)	4.7 ± 1.8 (15)	20.0 ± 3.3 (15)	10.7 ± 3.2 (15)	67.7 ± 6.9 (15)
MD4	5.8 (2)	53.9 ± 6.6 (3)	13.3 (2)	10.7 (2)	29.8 (2)	43.3 (2)
MM4xDD6	6.1 ± 1.5 (3)	47.3 ± 4.2 (3)	28.1 (2)	8.8 (2)	67.8 (2)	21 (2)
DD6	9.4 ± 1.3 (4)	17.1 ± 8.6 (4)	13.3 ± 0.6 (3)	10.7 ± 4.9 (3)	47.5 ± 9.0 (3)	32.5 ± 19.0 (3)
MδE1	5.6 ± 1.7 (4)	35.2 ± 4.8 (4)	13.3 ± 2.0 (3)	10.8 ± 1.0 (3)	48.5 ± 10.7 (3)	29.2 ± 2.5 (3)
MδE2	4.3 ± 2.1 (3)	22.8 ± 7.4 (3)	12.0 ± 4.2 (3)	11.6 ± 1.0 (3)	42.4 ± 21.2 (3)	26.6 ± 5.7 (3)
GG4:$RAG^{-/-}$	9.2 ± 8.4 (6)	53.4 ± 15.8 (11)	32.6 ± 13.2 (11)	11.9 ± 6.8 (11)	57.7 ± 8.5 (11)	22.6 ± 13.1 (11)
MG2:$RAG^{-/-}$	3.5 ± 1.6 (4)	46.4 ± 13.1 (5)	27.6 ± 9.6 (5)	12.4 ± 9.0 (5)	58.0 ± 4.1 (5)	18.2 ± 7.0 (5)
MM4:$RAG^{-/-}$	5.1 ± 0.6 (4)	49.3 ± 8.1 (11)	5.7 ± 4.6 (11)	19.9 ± 8.0 (11)	10.2 ± 8.5 (11)	35.0 ± 18.8 (11)
(B10.BRxC57BL/6)F_1	12.5 ± 3.4 (11)	0.1 ± 0.04 (7)	6.7 ± 2.6 (7)	46.5 ± 5.4 (9)	N/A	N/A

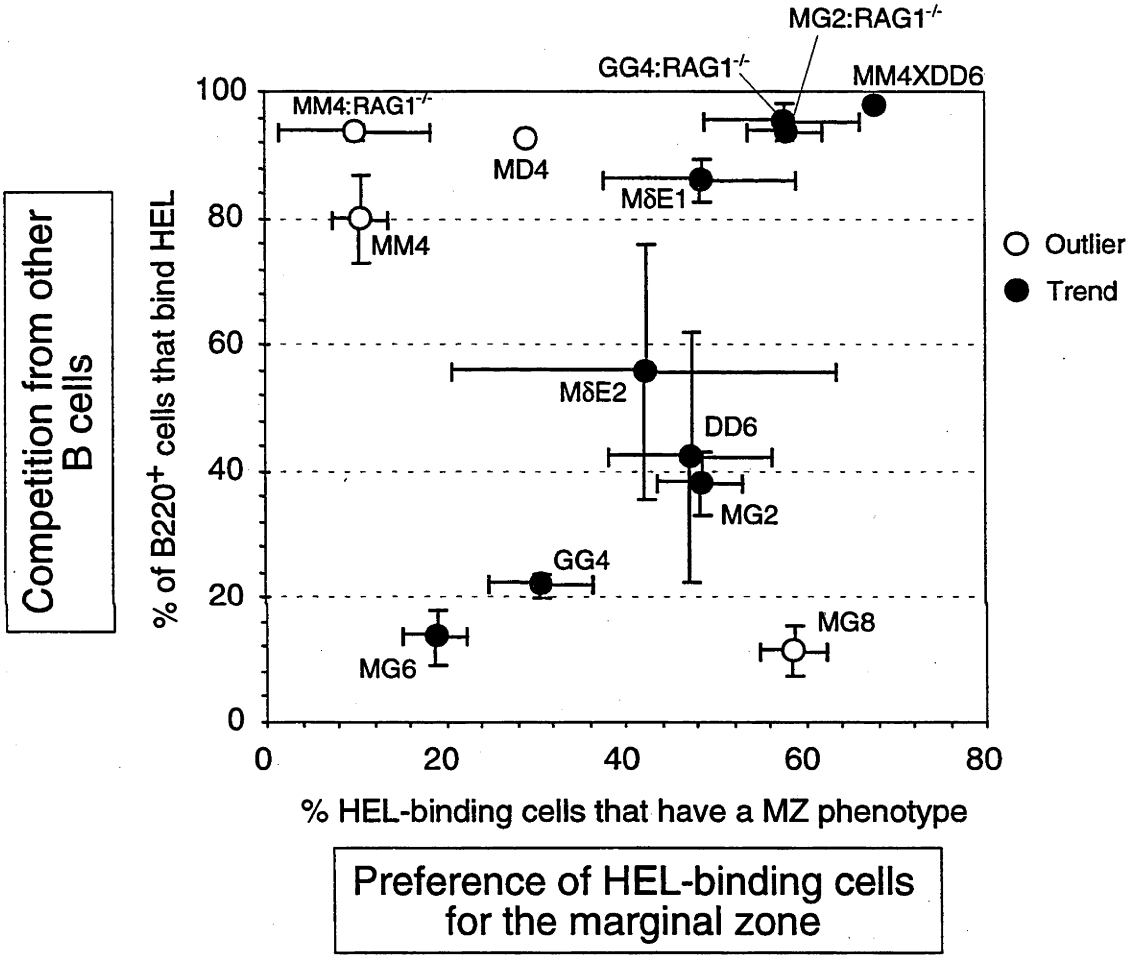
Figure 5-5. Correlating the effects of inter-clonal competition on entry into the marginal zone

To correlate the fraction of HEL-binding cells with a marginal zone phenotype with the level of clonal competition, the percentage of HEL-binding cells adopting a marginal zone phenotype (x-axis) was plotted against the percentage of $B220^+$ lymphocytes that bind HEL (y-axis) for each transgenic line used in this study.

The general trend, shown by points as filled circles, indicates that as the proportion of HEL-binding B cells increases, the proportion of HEL-binding marginal zone phenotype B cells also increases. In other words, as the level of clonal competition from non-HEL-binding B cells decreases, a greater fraction of HEL-binding B cells adopt a marginal zone phenotype.

Transgenic lines that do not follow the trend are shown by open circles. They include the IgM-only line MM4 on both the $RAG1^{+/+}$ and $RAG1^{-/-}$ background, the IgM/IgD line MD4 and the IgM/G line MG8.

Error bars are ± 1 standard error and are given for all lines with data points from >2 mice.


MG2:RAG1-/-
GG4:RAG1-/-
MM4XDD6
MM4:RAG1-/-
MD4
MδE1
MM4
MδE2
DD6
MG2
GG4
MG8
MG6
Outlier
Trend
Competition from other B cells
% of B220+ cells that bind HEL
% HEL-binding cells that have a MZ phenotype
0
20
40
60
80
100
Preference of HEL-binding cells for the marginal zone

Section 5.6. Summary

In this chapter the findings of Pogue and Goodnow (Pogue, 1996; Pogue, 2000) have been extended to show that differences in the level of CD21 expression amongst mature splenic B cells from the IgG, IgM/G and IgM HEL-transgenic lines reflects a differential recruitment of HEL-binding B cells into the marginal zone subset. This may be the result of two factors. On the one hand, BCR isotype seems to regulate marginal zone entry, as transgenic B cells expressing the IgG membrane tail form a greater fraction of the marginal zone compared to B cells expressing IgM alone. This is further evidence for differential signalling through the IgG isotype BCR during the formation of mature, peripheral HEL-binding B cells in IgG transgenic mice, along with previously reported receptor editing and receptor down-modulation phenomena (Pogue, 2000).

The role of BCR isotype signalling in allowing marginal zone recruitment is complicated, however, by the fact that inter-clonal B cell competition for marginal zone or mantle zone entry appears to also play a role. This has been noticed previously in both the HEL and 81x transgenic systems, where increasing proportions of "non-transgenic" endogenous B cells tend to lower the fraction of transgenic B cells that enter or accumulate in the marginal zone (Martin, 1997; Martin, 2000b). Thus far, competition has been removed in the IgM, IgM/G and IgG transgenic anti-HEL lines by crossing them to $RAG1^{-/-}$. When there are no competing B cells, both the IgG and IgM/G transgenic lines have an equivalent, high proportion of HEL-binding cells in the marginal zone subset, while HEL-binding cells in the IgM transgenic line retain a predominantly follicular phenotype. A more useful way of modelling clonal competition in the future would be the construction of competition bone marrow chimeras, as these will retain intact adaptive immune systems, unlike $RAG1^{-/-}$ mice.

Expression of the IgG membrane tail is not the only feature that allows increased marginal zone accumulation by HEL-binding B cells. For example, in MM4xDD6 mice that co-express IgM and IgD, over 60 % of HEL-binding B cells have a marginal zone phenotype. The analysis of these other transgenic lines is complicated, however, because of the variable effects of inter-clonal competition. In general, as the proportion of HEL-binding B cells in the spleen is increased, the fraction of HEL-binding cells that have a marginal zone phenotype is increased (see Figure 5-5). It may be that the transgenic lines that follow this trend (GG4, MG2, MG6, MG8, DD6, MδE1, MδE2 and MM4xDD6) carry

BCR isotype combinations that are equally able to drive recruitment of HEL-binding B cells into the marginal zone subset. This possibility can only be tested, however, by normalising the levels of competing "non-transgenic" B cells across each line (see Section 5.7).

In summary, this chapter has presented new evidence for differential signalling through IgG and IgM isotype BCRs in the formation of different peripheral B cell subsets. Mice expressing the IgG membrane tail are able to support a much greater fraction of HEL-binding marginal zone B cells compared to mice bearing IgM alone. It is unclear at present whether this is a unique feature of the IgG isotype BCR, as other combinations of IgM and IgD isotype receptors also allow accumulation of HEL-binding marginal zone B cells. In all of these analyses the action of BCR isotype in allowing marginal zone entry and accumulation is complicated by the variable effects of inter-clonal competition.

Section 5.7. Future work

In the future it will be important to address the issue of inter-clonal competition in determining marginal zone entry using competition bone marrow chimeras to normalise the number of competing endogenous B cell. Varying mixtures of Ig transgenic and non-transgenic bone marrow could be used to reconstitute irradiated recipient mice. By comparing mice with similar levels of "competing" non-transgenic B cells the ability of each BCR isotype to drive marginal zone recruitment could be more accurately assessed. A reciprocal study where each transgenic line was crossed to the $RAG1^{-/-}$ background would also allow the degree of marginal zone formation to be assessed in the absence of competition. This may not be as useful as bone marrow chimeras, as the interpretation of RAG experiments is complicated because it introduces new variables by the removal of T cells, natural antibody and a loss of gut adaptive immunity.

It will also be important to determine whether the presence of transgenic cells in the marginal zone subsets of the various transgenic lines is due to differences in the rate of production of marginal zone cells or differences in their lifespan after marginal zone entry. This could be done by BrdU labelling or by the transfer of immature B cells into $RAG1^{-/-}$ recipients to track their differentiation into the various mature B cell compartments.

Finally, it will be important to address the signals downstream from the HEL-binding BCRs of the various lines that allow recruitment into the marginal zone subset. Candidate signalling molecules include Btk, CD19 (Martin, 2000b), Aiolos (Cariappa, 2001) and NFκB p50 (Cariappa, 2000).

Chapter 6. Line to line variation does not explain differential B cell responses to antigen *in vivo*

Section 6.1. Introduction

The previous chapters have demonstrated that transgenic B cells from a variety of lines bearing the IgG membrane tail make a more robust response to antigen than transgenic B cells bearing combinations of IgM or IgD isotype BCRs. The hypothesis presented in this thesis is that differential responses to antigen by transgenic lines bearing the IgG membrane tail is a reflection of isotype-specific signalling *via* IgG.

Despite these observations, the assumption that BCR isotype is the only difference between the Ig transgenic lines used in this study is not appropriate for several reasons.

At the most fundamental level, line to line variation due to transgene integration site and transgene copy number differences are a source of variation. As described previously (Pogue, 2000) there are also significant differences in peripheral B cell phenotypes between the IgM-only, IgG and IgM/G transgenic lines, such as receptor editing and BCR modulation that correlate with transgene copy number.

In addition, the transgenic constructs used to make the different lines vary in both the extent and the type of switch region included upstream from the constant region gene elements. Although isotype switching is not a normal event in most Ig transgenic systems due to the Hc transgene integrating in a location separate from the IgH locus, it is possible that switch recombination could initiate at the transgene locus and lead to chromosome exchanges or breakage, resulting in cell death.

Finally, as described in the Chapter 5, differences in B cell development between transgenic lines do not simply alter the fraction of peripheral B cells that bind HEL. Large variations in the proportion of mature HEL-binding B cells with marginal zone versus follicular phenotypes are also apparent. This is a particularly interesting finding, as marginal zone B cells show evidence of a pre-activated phenotype and may respond more robustly than follicular B cells to certain types of antigen (Liu, 1991; Martin, 2001). In particular, IgG and IgM/G transgenic lines have a much greater fraction of HEL-binding MZ cells compared to the IgM-only line, providing a potential explanation for the differences in reactivity to antigen on adoptive transfer that were described in Chapter 3 and Chapter 4.

This chapter describes a range of experiments designed to test whether differences other than BCR isotype could explain the pattern of reactivity amongst transgenic lines to antigen *in vivo*.

Section 6.2. Line to line variation in construct integration site, transgene copy number or transgene switch regions does not explain different responses to antigen

Figure 6-1 shows the results of two experiments comparing GG4, MG2, MG6, MG8, MM4, MδE1, MδE2, DD6 and MM4xDD6 transgenic lines. Recipient animals received 10^6 Ig transgenic B cells, 10^6 TCR transgenic T cells and immunising antigen in adjuvant. On day 5 after transfer B cell clonal expansion was assessed by FACS, and AFC production measured by ELISPOT. Numerous comparisons can be made from these experiments to test the role of line to line variation in transgenic B cell responses to antigen.

Firstly, comparisons were made between three independent IgM/G lines (MG2, MG6 and MG8) produced with the same transgenic construct, as well as between two independent IgMδE lines (MδE1 and MδE2), again produced using the same construct. As shown in Figure 3-4 and Figure 6-1, all MG lines produced a similar number of AFCs and underwent a similar degree of clonal expansion. Similarly, both MδE lines were comparable by these criteria, although making a smaller response. This shows that independent transgenic lines made with the same construct gave a consistent pattern of reactivity to antigen *in vivo*, and that the effects of random transgene integration are unlikely to explain the variations between the various Ig transgenic lines *in vivo*.

Secondly, from this experiment it was possible to compare transgenic lines that contained different transgene switch regions (see Table 6-1 and Figure 2-1). Switch regions are GC-rich simple tandem repeat DNA sequences of 1-10 kb length found upstream of constant region gene segments (except δ) (Stavnezer, 2000), and were included in the transgenic constructs used to make the panel of mice. The MM4 (Brink, 1992) and MD4 (Goodnow, 1988; Goodnow, 1989) lines contained intact μ switch regions, while the MG (Pogue, 2000) and MδE lines (Pogue, 1994) all had an identical partial μ switch region containing a 2.8 kb internal deletion. The GG4 line (Pogue, 2000) contained a hybrid μ/γ1 switch region, and the DD6 line lacked a switch region (Brink, 1992). The impact of this variation on B cell responses to antigen *in vivo* was not clear. While interchromosomal switch recombination between the endogenous IgH locus and the transgene is considered a rare event (Durdik, 1989), one of the outcomes of the induction of switch recombination at the transgene locus might be the introduction of chromosomal breakages (Kenter, 1999), leading to cell death. It was not known whether the complete transgenic μ switch regions might present a better target for the recombination machinery than partial μ switch regions.

Also, it was possible that μ/γ1 hybrid switch regions were targeted differently to μ switch regions. It was therefore important to investigate this as a cause of differential clonal expansion and cell loss amongst the transgenic lines during an immune response to antigen *in vivo*.

Switch region variation can be ruled out as a cause of differential responses by several observations. Firstly, the GG4 and MG lines contain different switch regions yet make comparable responses to antigen. In GG4, the switch region is a μ/γ1 hybrid, whereas in the MG lines it is a partial μ switch region. Secondly, each MG and MδE line carries an identical partial μ switch region, yet the MG lines consistently make at least 10-fold more AFCs and a 10-fold greater clonal expansion response to antigen compared to the MδE lines. Similarly, both the MM4 and MD4 transgenic constructs carry a complete μ switch region. Despite this, transgenic B cells from the MD4 line consistently make more robust responses to antigen compared to the MM4 line (see Figure 3-3). Finally, the reactivity of the MM4 line is "rescued" by crossing to the DD6 line, and MM4xDD6 transgenic B cells now make a comparable response to antigen as lines carrying the membrane tail of IgG. This is contrary to the expected result if improper switch recombination at the μ transgene was a major determinant of reactivity *in vivo*. In the MM4xDD6 line, the IgM transgene is still transcriptionally active (Bell, S. and Goodnow, C., unpublished observations), despite IgM protein being out-competed by IgD for expression as the predominant surface isotype. This suggests that switch recombination at the transgenic μ switch region would still be induced at the same rate in the MM4xDD6 line compared to the MM4 line. As the MM4xDD6 transgenic B cells make a robust response to antigen, switch recombination at the transgenic μ locus is unlikely to be responsible for the cell loss observed with the MM4 line.

Collectively, these results demonstrate that variations in the transgene integration sites and transgene switch regions of the various transgenic lines does not explain the pattern of antigen reactivity seen *in vivo*.

Table 6-1. The type and extent of switch regions used in each transgenic construct

Transgenic Line	Heavy chain constant region	Switch region
GG4	gamma	μ/gamma hybrid
MG2, MG6, MG8	μ/gamma hybrid	μ*
MM4	μ	μ
MD4	μ and delta	μ
MdE1, MdE2	μ/delta hybrid	μ*
DD6	delta	none
MM4xDD6	μ and delta (independent)	μ*

Abbreviations used: μ, mu heavy chain constant region or switch region; μ*, mu switch region carrying a 2.8 kb internal deletion

References: GG4, MG2, MG6, MG8 (Pogue, 1996; Pogue, 2000); MM4, DD6 (Brink, 1992); MD4 (Goodnow, 1988; Goodnow, 1989); MδE1, MδE2 (Pogue, 1994)

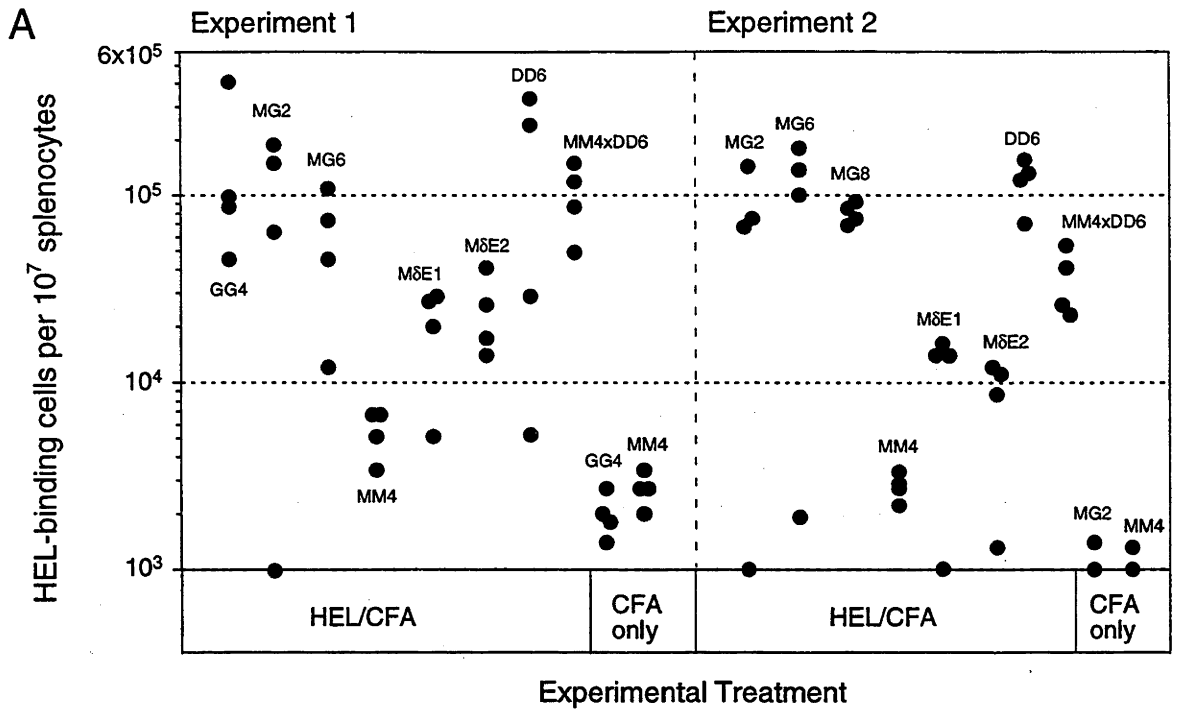
Figure 6-1. A comparison of immune responsiveness between transgenic B cells from lines GG4, MG2, MG6, MG8, MδE1, MδE2, MM4, DD6 and MM4xDD6

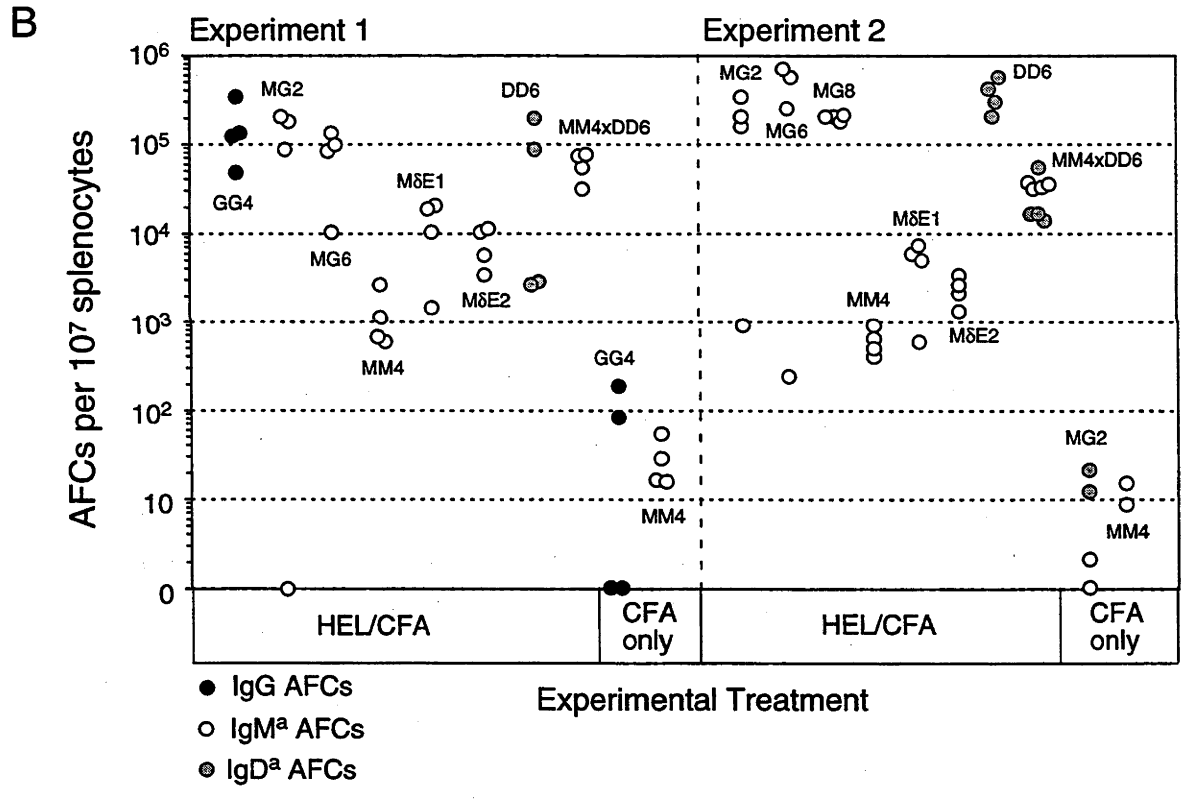
In both transfer experiments outlined in (A) and (B), 10^6 transgenic B cells of the indicated type were transferred along with 10^6 TCR transgenic T cells into (B10.BRxC57BL/6)F_1 recipient mice immunised at the same time with HEL in CFA or CFA alone. On day 5 after transfer, spleen samples were analysed by FACS and ELISPOT to enumerate the number of HEL-binding cells and the number of anti-HEL AFCs.

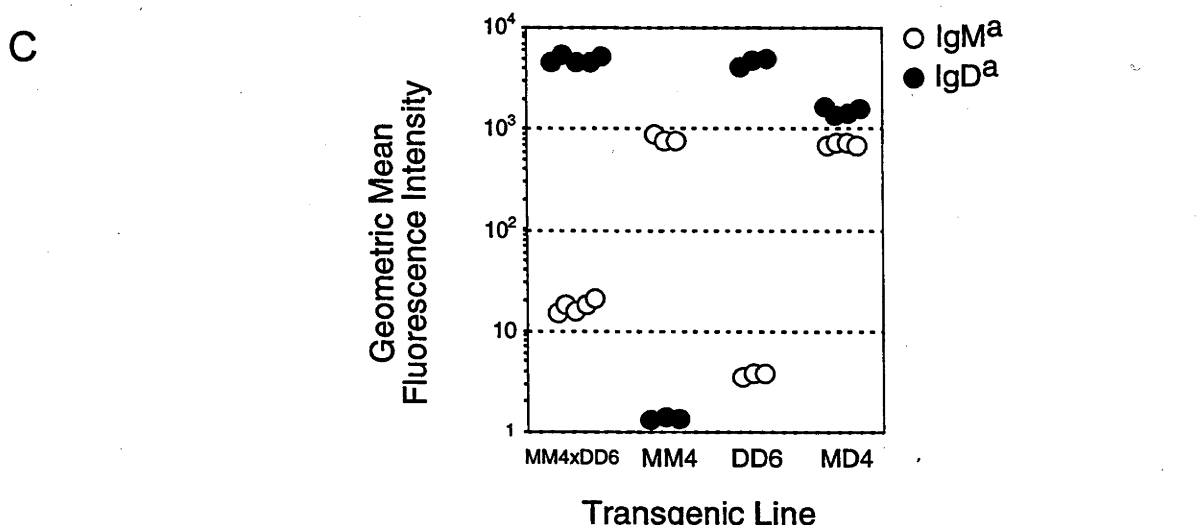
A. Clonal expansion response as measured by FACS for both experiments. Data is shown as the number of HEL-binding lymphocytes per 10^7 recipient lymphocytes.

B. Anti-lysozyme IgG_1, IgD^a or IgM^a AFC responses as measured by ELISPOT for both experiments.

C. Graph showing the geometric mean fluorescence intensity of IgM^a (open circles) and IgD^a staining (filled circles) on $B220^+$ peripheral blood lymphocytes from MM4xDD6, MM4, DD6 and MD4 transgenic mice. Each point represents a separate mouse. Data in this panel was acquired during a single phenotyping experiment and all the blood samples were stained identically using the same batch of reagents.


A
Experiment 1
Experiment 2
6x10^5
10^5
10^4
10^3
HEL-binding cells per 10^7 splenocytes
MG2
MG6
DD6
MM4xDD6
GG4
MδE1
MδE2
MM4
MG8
HEL/CFA
CFA only
Experimental Treatment


B
Experiment 1
Experiment 2
10^6
10^5
10^4
10^3
10^2
10
0
AFCs per 10^7 splenocytes
MG2
MG6
MG8
DD6
MM4xDD6
GG4
MδE1
MδE2
MM4
HEL/CFA
CFA only
IgG AFCs
IgM^a AFCs
IgD^a AFCs
Experimental Treatment


C
10^4
10^3
10^2
10
1
Geometric Mean Fluorescence Intensity
IgM^a
IgD^a
MM4xDD6
MM4
DD6
MD4
Transgenic Line

6.2 (a) Variations in the proportion of HEL-binding B cells do not explain different responses to antigen

The high affinity of the anti-HEL BCR for antigen allows the unequivocal staining of cells bearing a high density of transgenic BCR by FACS (Townsend, 2001). In contrast to other anti-HEL lines, the GG4 and MG lines (MG2, MG6 and MG8) accumulate a significant population of non-HEL-binding B cells in the periphery, apparently the result of editing (Pogue, 2000). High-affinity HEL-binding cells in these lines are negative for IgD expression, suggesting effective allelic exclusion in the cells that retain HEL-binding. The transfer experiments in this study mostly used whole, unfractionated spleen suspensions and relied on high-affinity HEL-binding as a measure of the number of "transgenic" B cells in the transfer inoculum, but it is difficult to completely rule out some low-level of expression of anti-HEL BCR on non-HEL-binding "edited" B cells. If some non-HEL-binding B cells in the lines expressing the IgG membrane tail retained a low level expression of the transgenic receptor, it may be that a greater number of antigen-reactive cells were transferred into recipient mice, leading to their apparently greater response to antigen *in vivo*.

To exclude this possibility, GG4, MG2 and MM4 lines were crossed to the $RAG1^{-/-}$ background. As seen in Figure 5-4 and Figure 6-3, all the splenic and lymph node B cells in the GG4, MG2 and MM4 lines on the $RAG1^{-/-}$ background are high affinity HEL-binding cells. In one experiment, $RAG1^{-/-}$ transgenic B cells were co-transferred into an anti-lysozyme immune response in C57BL/6 mice that had been previously primed with lysozyme in CFA to expand endogenous helper T cells. Figure 6-2 shows that IgG:$RAG1^{-/-}$ and IgM/G:$RAG1^{-/-}$ B cells make a similar number of AFCs, while IgG:$RAG1^{-/-}$ B cells make ~10-fold more AFCs than IgM:$RAG1^{-/-}$ B cells. This is an identical result to transfer experiments conducted with $RAG1^{+/+}$ Ig transgenic lines, and shows that B cells bearing the membrane tail of IgG make an enhanced response to antigen on a per cell basis even in the absence of non-HEL-binding peripheral B cells. The same result was obtained by the adoptive transfer of IgG:$RAG1^{-/-}$, IgM/G:$RAG1^{-/-}$ and IgM:$RAG1^{-/-}$ transgenic lymph node B cells, where IgG and IgM/G $RAG1^{-/-}$ B cells made similar AFC responses that were 100-fold greater than an equivalent number of IgM:$RAG1^{-/-}$ B cells (Figure 6-3).

Collectively, these data rule out the possibility that non-HEL-binding B cells contribute to the pattern of antigen reactivity described in this work.

Section 6.3. Marginal zone and follicular subsets differ between lines but do not correlate with reactivity to antigen

The previous chapter documented the wide variation in HEL-binding marginal zone and follicular subsets in the spleens of the various Ig transgenic lines used in this study. Numerous properties have been attributed to marginal zone B cells, including a pre-activated phenotype (Liu, 1995; Martin, 2000a; Martin, 2001) and enhanced APC abilities (Liu, 1995), as well as rapid, predominantly extrafollicular antibody responses against various antigens (Liu, 1991; Martin, 2001). So, while an equivalent number of splenic HEL-binding cells were used from each line for adoptive transfer, there were considerable differences in the fraction of these HEL-binding cells with marginal zone versus follicular phenotypes. This was an attractive explanation for differences in reactivity between, for example GG4, MG2 and MM4 lines. Both GG4 and MG2 have a much greater fraction of marginal zone phenotype HEL-binding cells (30.9 ± 5.8 % and 48.6 ± 4.9 % respectively) compared to the MM4 line (10.7 ± 3.2 %), and this seems to correlate with the reactivity to antigen seen during adoptive transfer.

To test whether variations in the marginal zone and follicular phenotypes of transgenic B cells from the various lines was responsible for their varying reactivity to antigen *in vivo*, four experimental approaches were taken.

6.3 (a) The proportions of MZ versus FO subsets in different lines do not correlate with responses to antigen

Data presented in Chapter 5, in conjunction with the experiments described in Figure 6-1 suggest that marginal zone versus follicular subset differences between the transgenic lines do not correlate with B cell reactivity *in vivo* on adoptive transfer.

In terms of reactivity to antigen, from Figure 6-1 and Figure 3-3 it is possible to rank each transgenic line from highest to lowest:

GG4 ~ MG2 ~ MG6 ~ MG8 ~ DD6 > MM4xDD6 > MD4 ~ MδE1 ~ MδE2 > MM4

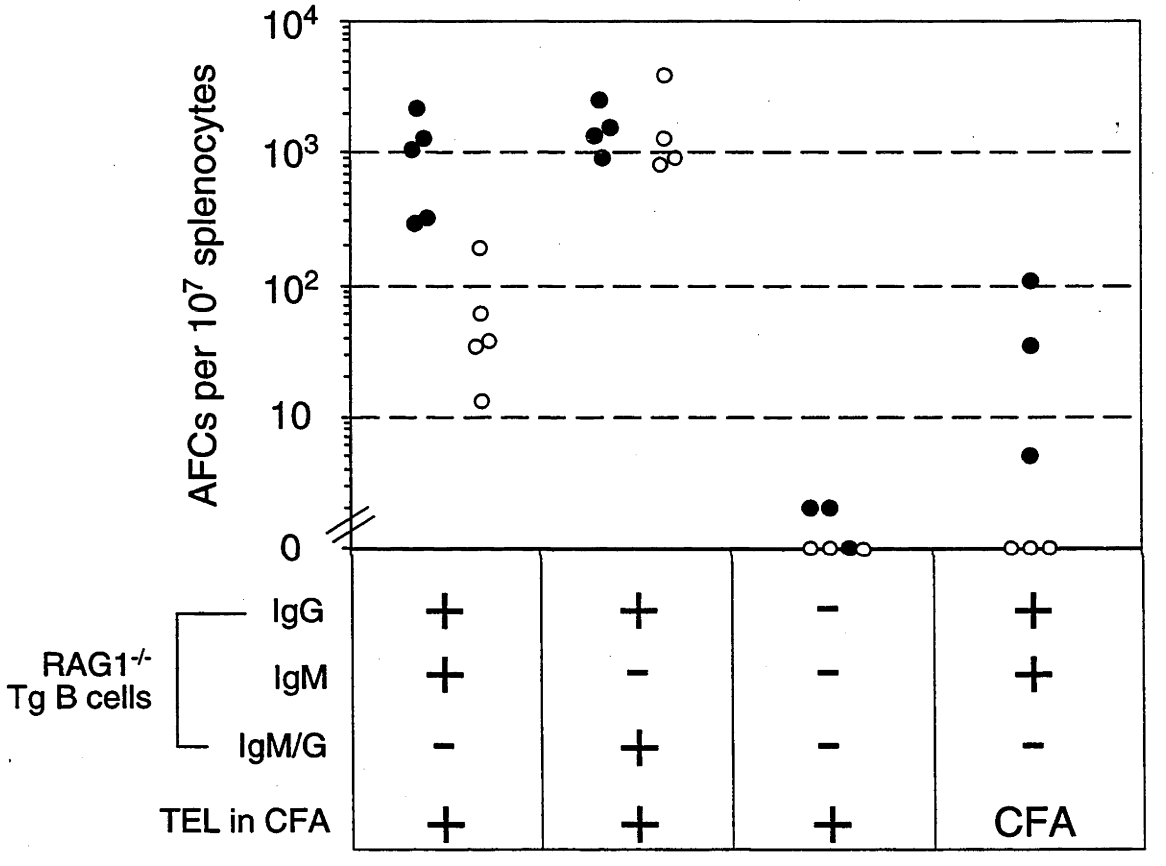
In terms of the fraction of HEL-binding cells that have a marginal zone phenotype, it is also possible to rank B cells from each transgenic line (Table 5-1):

MM4xDD6 > MG8 > MG2 ~ MδE1 ~ DD6 ~ MδE2 > GG4 ~ MD4 > MG6 > MM4

Figure 6-2. Transgenic B cells on the RAG1$^{-/-}$ background show the same pattern of reactivity to antigen *in vivo*

10^6 IgG:RAG1$^{-/-}$ transgenic B cells were mixed in a 1:1 ratio with either 10^6 IgM:RAG1$^{-/-}$ or 10^6 IgM/G:RAG1$^{-/-}$ transgenic B cells. These were injected i.v., along with a boost of 50 μg soluble TEL into C57BL/6 recipients that had been primed with 100 μg TEL in CFA 7 days previously.

On day 5 after immunisation and transfer spleen samples were taken and ELISPOT assays performed to enumerate anti-lysozyme AFCs. The results are given as the number of anti-lysozyme IgG_1 AFCs (closed circles) and the number of anti-lysozyme IgM^a AFCs (open circles) per 10^7 recipient splenocytes.


AFCs per 10^7 splenocytes
10^4
10^3
10^2
10
0
RAG1$^{-/-}$ Tg B cells
IgG
IgM
IgM/G
TEL in CFA
+
+
-
+
+
-
+
+
-
-
+
-
+
+
+
CFA

In some cases, the fraction of marginal zone phenotype HEL-binding cells correlates well with the reactivity of a particular line. For example the MM4 line has a comparatively small fraction of HEL-binding marginal zone cells, and B cells from this line consistently make the poorest response to antigen *in vivo*. However there are some notable exceptions. For example both the MG6 and GG4 lines have comparatively low fractions of HEL-binding MZ phenotype B cells, yet these consistently produce the most robust immune responses *in vivo*. In addition, while the MδE1 and MδE2 lines have a comparable proportion of HEL-binding marginal B zone cells compared to the MG2 line, they consistently produce ~100-fold fewer anti-HEL AFCs and undergo at least a 10-fold lower clonal expansion response compared to the MG2 line.

Due to these inconsistencies between the observed reactivity of each line and the fraction of HEL-binding cells that have a marginal zone phenotype, it is clear that differences in the partitioning of HEL-binding cells into one peripheral subset or another is not the only factor affecting B cell responses to antigen.

6.3 (b) Immature sorted B cells retain differential responses to antigen

To directly examine whether differences in the mature B cell subsets are required for differential responses to antigen, immature bone marrow B cells from IgG (GG4) and IgM-only (MM4) transgenic lines were sorted on the basis of HEL-binding and a lack of CD21 expression. CD21 expression gradually increases during B cell ontogeny, and $CD21^-$ bone marrow cells are immature (Gelfand, 1974; Ahearn, 1989). The sorted immature B cell bone marrow subsets from the IgG and IgM transgenic lines show no evidence of selection, and form a similar proportion of bone marrow lymphocytes in both cases (Pogue, 1996; Pogue, 2000).

Figure 6-3 part A shows the results of the transfer experiment using the sorted immature cells. Representative FACS plots of HEL-binding and CD21 expression on bone marrow lymphocytes from IgG and IgM donor transgenic mice show that the $CD21^-$ HEL-binding subsets are identical in frequency and surface Ig receptor levels. When seeded into immunised recipients along with TCR transgenic T cells, IgG isotype immature HEL-binding B cells still produced ~100-fold more AFCs on a per cell basis compared to IgM isotype immature HEL-binding B cells. This demonstrates that identical B cell populations that differ only in BCR isotype expression but show no evidence for selection still produce differential responses to antigen on adoptive transfer. This argues that the selective differences in splenic peripheral B cell subsets are not necessary for differential responses to antigen in this system.

6.3 (c) IgG, IgM/G and IgM transgenic lymph node B cells retain differential responses to antigen

Lymph node B cells from IgG, IgM/G and IgM RAG1-deficient mice show no evidence of selection (Figure 6-3). These provided an identically-matched pool of mature B cells with which to further test the role of selection in B cell responses to antigen. As shown in Figure 6-3 part B, lymph node cells from these lines are homogeneous $B220^{+}$ HEL-binding cells, with a predominantly follicular phenotype on the basis of CD21 and CD23 expression.

Mature lymph node B cells from IgG:$RAG1^{-/-}$, IgM/G:$RAG1^{-/-}$ and IgM:$RAG1^{-/-}$ mice were transferred along with TCR transgenic T cells into immunised recipients. As shown in Figure 6-3 part B, lymph node-derived transgenic B cells that bear the IgG membrane tail make a 100-fold greater AFC response compared to lymph node-derived transgenic B cells with IgM isotype receptors.

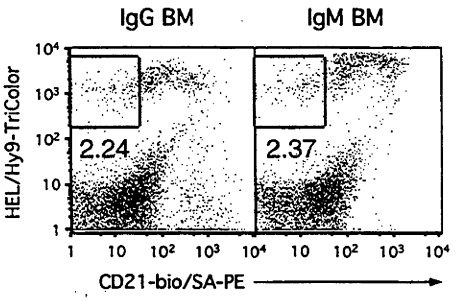
This is further evidence that differences in marginal zone versus follicular subset partitioning in the spleen is not necessary to explain differential reactivity to antigen in this system. In this case phenotypically equivalent mature B cells still retain differential responses to antigen, and the only factor determining their response is the presence or absence of the IgG membrane tail.

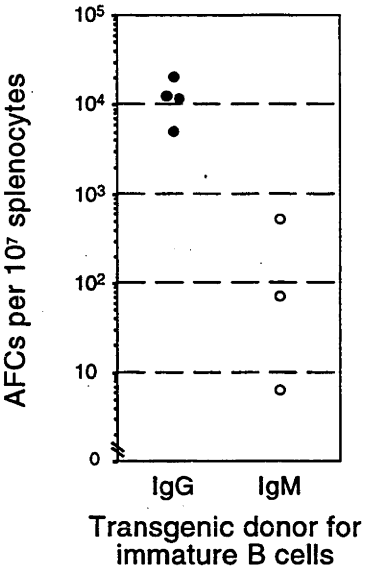
Figure 6-3. Immature bone marrow B cells and mature lymph node B cells from different transgenic lines retain differential responses to antigen

A. Immature phenotype $CD21^-$ HEL-binding B cells were sorted from IgG and IgM transgenic bone marrow. FACS plots show CD21 expression (x-axis) and HEL-binding (y-axis) on bone marrow lymphocytes from IgG and IgM transgenic mice. Gates indicate the sorted populations and numbers give the fraction of bone marrow lymphocytes that are $CD21^-$ and HEL-binding in both samples. 10^5 sorted B cells were transferred along with 10^5 TCR transgenic T cells into immunised $(B10.BRxC57BL/6)F_1$ recipients, and the production of anti-lysozyme IgG_1 (filled circles) and IgM^a (open circles) AFCs measured on day 5 by ELISPOT. Data is given as the number of anti-lysozyme AFCs per 10^7 recipient splenocytes.

B. Lymph node cells from IgG:$RAG1^{-/-}$, IgM/G:$RAG1^{-/-}$ and IgM:$RAG1^{-/-}$ transgenic mice were used as a source of donor B cells. As shown by FACS dot plots, B cells from all of these lines represent a homogeneous population of $B220^+$ HEL-binding cells that have a predominantly $CD21^{med}$ $CD23^+$ follicular phenotype. No $CD21^{hi}$ $CD23^-$ cells were present in the samples (gates were set on spleen samples analysed in parallel). 10^6 HEL-binding lymph node cells of each type were transferred along with 10^6 TCR transgenic T cells into immunised B10.BR recipient mice. On day 5 after transfer the production of anti-lysozyme AFCs in the spleen of recipient mice was measured by ELISPOT. Anti-lysozyme IgG_1 AFCs are given by filled circles and anti-lysozyme IgM^a AFCs are given by open circles.

A


IgG BM
IgM BM
HEL/Hy9-TriColor
2.24
2.37
CD21-bio/SA-PE


AFCs per 10⁷ splenocytes
IgG
IgM
Transgenic donor for immature B cells

B

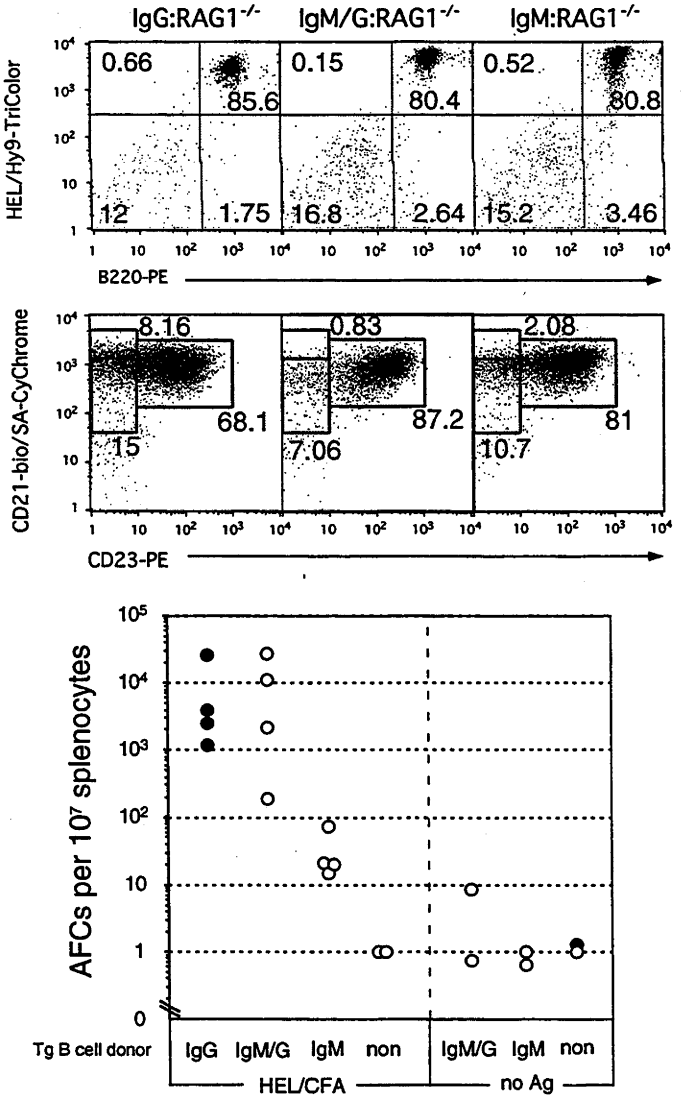
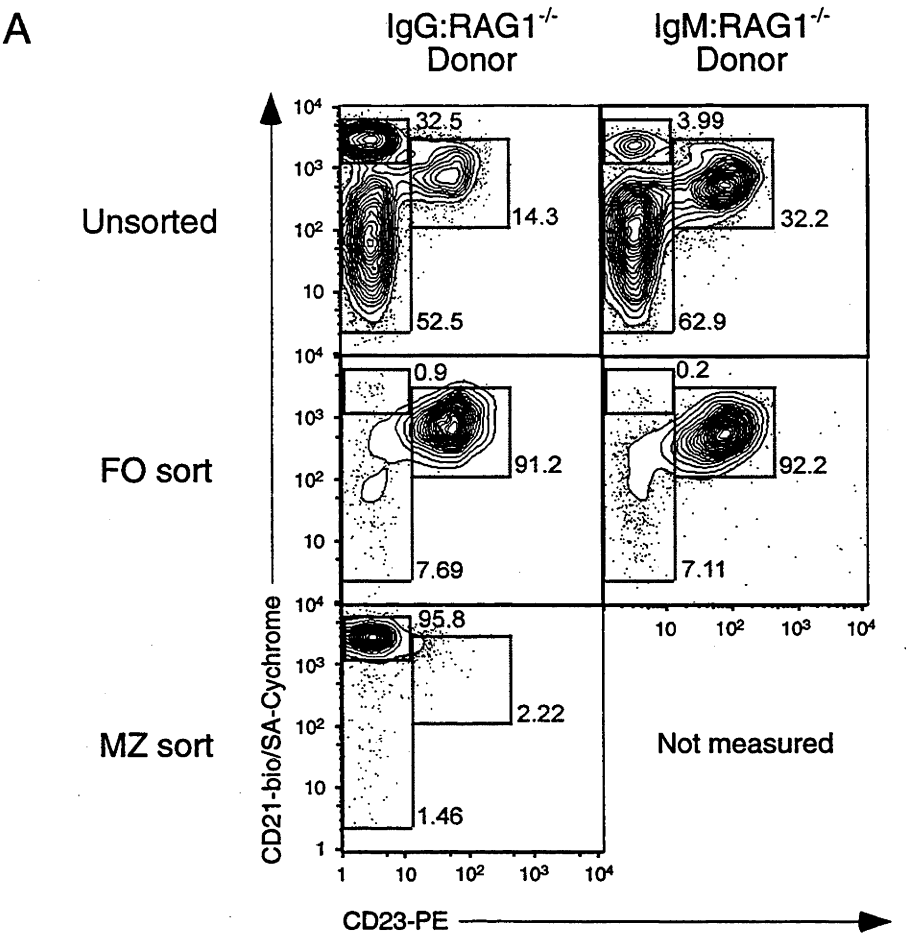

IgG:RAG1-/-
IgM/G:RAG1-/-
IgM:RAG1-/-
HEL/Hy9-TriColor
0.66
85.6
12
1.75
0.15
80.4
16.8
2.64
0.52
80.8
15.2
3.46
B220-PE
CD21-bio/SA-CyChrome
8.16
68.1
15
0.83
87.2
7.06
2.08
81
10.7
CD23-PE
AFCs per 10⁷ splenocytes
Tg B cell donor
IgG
IgM/G
IgM
non
IgM/G
IgM
non
HEL/CFA
no Ag

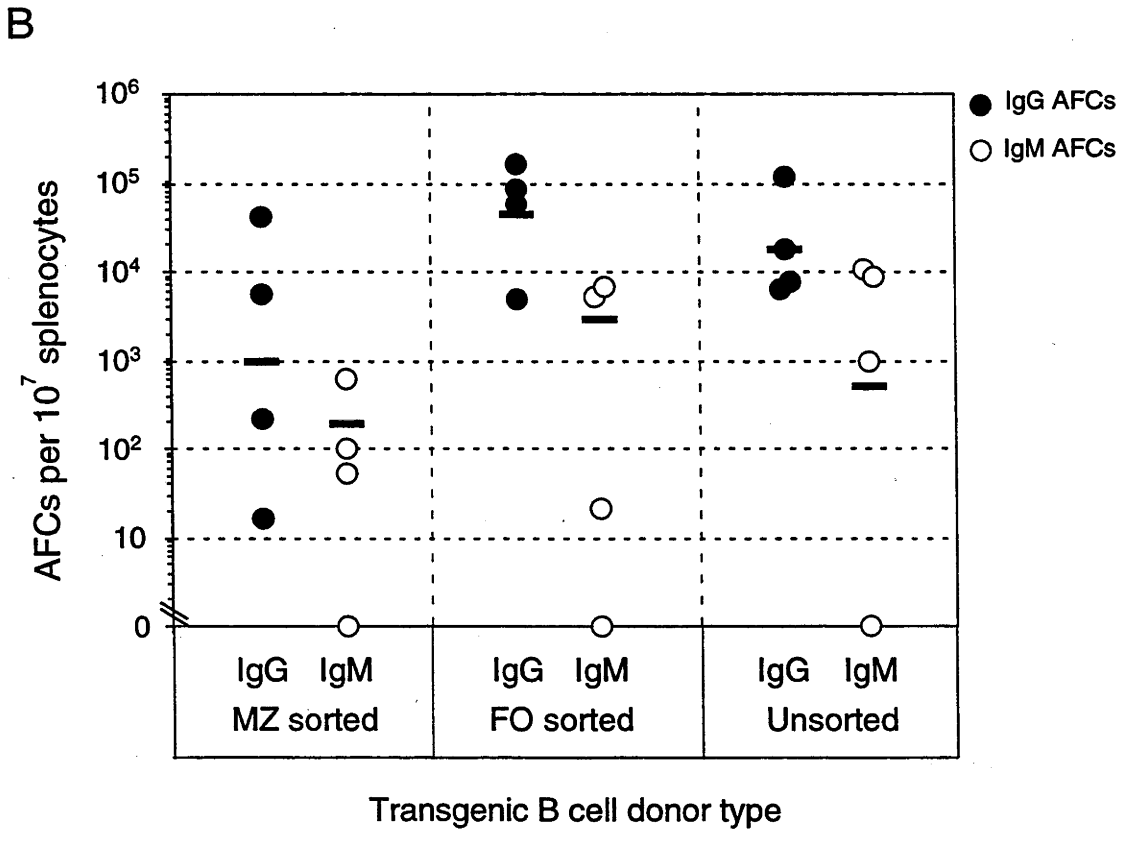
Figure 6-4. Sorted marginal zone and follicular phenotype spleen cells from IgG or IgM RAG1$^{-/-}$ mice retain differential responses to antigen

Marginal zone phenotype (CD21^{hi} CD23^{-}) and follicular phenotype (CD21^{med} CD23^{+}) B cells were sorted from IgG:RAG1$^{-/-}$ and IgM:RAG1$^{-/-}$ transgenic donor mice.

FACS contour plots are gated on lymphocytes by scatter, and show unsorted, sorted marginal zone and sorted follicular phenotype spleen cells from IgG and IgM RAG1$^{-/-}$ transgenic mice. The gates used to sort the marginal zone and follicular subsets are indicated on each plot, as well as post-sort samples showing the purity of the sorted cells.

10^5 of each type of sorted or unsorted transgenic B cell was transferred along with $2x10^5$ TCR transgenic T cells into immunised (B10.BRxC57BL/6)F_1 recipients. On day 5 after immunisation and transfer the number of AFCs was determined by ELISPOT. Bars show the geometric mean for each experimental group.


A
IgG:RAG1-/- Donor
IgM:RAG1-/- Donor
Unsorted
FO sort
MZ sort
32.5
14.3
52.5
3.99
32.2
62.9
0.9
91.2
7.69
0.2
92.2
7.11
95.8
2.22
1.46
Not measured
CD21-bio/SA-Cychrome
CD23-PE


B
IgG AFCs
IgM AFCs
AFCs per 10^7 splenocytes
IgG IgM
MZ sorted
IgG IgM
FO sorted
IgG IgM
Unsorted
Transgenic B cell donor type

6.3 (d) Sorted MZ versus FO subsets retain differential responses to antigen

To directly test the reactivity of the different splenic peripheral B cell to antigen, marginal zone and follicular peripheral B cell subsets were sorted (Figure 6-4).

For this experiment, IgG:RAG1$^{-/-}$ and IgM:RAG1$^{-/-}$ transgenic mice were used as a source of transgenic B cells. These RAG1$^{-/-}$ IgG and IgM transgenics show large differences in marginal zone and follicular B cell partitioning. As all peripheral B cells are HEL-binding (see Figure 5-4), it was not necessary to stain and sort on the basis of BCR specificity. Marginal zone and follicular B cells were sorted from IgG and IgM RAG1$^{-/-}$ transgenics on the basis of CD21 and CD23 expression. Also, IgG:RAG1$^{-/-}$ and IgM:RAG1$^{-/-}$ spleen samples were stained and treated identically to the sorted fractions, except they did not pass through the flow cytometer. These were transferred into immunised recipients at the same time as the sorted subsets, to control for the effects of CD21 and CD23 ligation on cell viability and activation status. After sorting from IgG and IgM transgenic donors, 10^5 MZ or FO phenotype B cells were transferred into immunised recipients along with 2 x 10^5 TCR transgenic cells.

Figure 6-4 shows the results of this experiment as the number of anti-HEL AFCs per recipient on day 5 after immunisation and transfer. In all groups the IgG transgenic B cells produced more AFCs on average compared to the corresponding IgM transgenic B cells. This experiment is difficult to interpret, however, as there were large variations in the AFC response within each experimental group. This may be due to the relatively low numbers of B cells transferred, resulting in variable loss upon injection and variable incorporation into lymphoid tissues. Also, the effects of staining marginal zone and follicular subsets with antibodies that cross-link CD21 and CD23 may have influenced both the viability and the activation status of the cells prior to transfer. For example, the sorted marginal zone subsets made a poorer AFC response compared to the sorted follicular subsets, possibly as a result of crosslinking CD21 which is more highly expressed on marginal zone B cells (Loder, 1999) and might differentially affect cell viability. Alternatively, marginal zone B cells are non-recirculating cells (Kumararatne, 1981a; Kumararatne, 1981b) which may not home from the blood to the spleen efficiently, thereby reducing the observed response in those groups. In the future it should be possible to repeat this experiment, perhaps using alternative sorting markers such as CD1d (Roark, 1998), as well as transferring 5 or 10 times more transgenic B cells per recipient to unequivocally confirm these results.

Section 6.4. The effect of IgD expression on transgenic B cell responses to antigen *in vivo*.

Throughout this study, B cells from Ig transgenic mice expressing the IgG membrane tail have made robust responses to antigen *in vivo*. The responses of the various lines expressing combinations of IgM and IgD isotype receptors have been more variable. There are several possibilities for the source of this variation. There are differences in transgenic construct types, line-to-line variation due to transgene integration site and differences in the development and surface phenotype of peripheral B cells in these lines, although none of these parameters correlate perfectly with responses to antigen *in vivo*. Another possibility is that these variations are a real reflection of isotype-specific signalling through IgM and IgD antigen receptors in the context of the anti-HEL transgenic system.

The IgM-only MM4 line consistently makes fewer AFCs and a poorer clonal expansion response compared to the IgM/IgD co-expressing line MD4 (see Figure 3-3). To extend this observation, the IgM-only line MM4 was also compared to lines MδE1, MδE2, DD6 and MM4xDD6 using adoptive transfer with TCR transgenic T cells into immunised recipients. Figure 6-1 shows results from two such experiments.

Consistent with previous findings, the IgM-only line MM4 made a three-fold poorer clonal expansion response compared to either IgMδE line. Interestingly, the IgD-only line DD6 made a similar response to the IgG line GG4 and the IgM/G lines MG2, MG6 and MG8, both in terms of clonal expansion and AFC production. Co-expression of transgenic IgD from the DD6 transgene with IgM from the MM4 transgene in the (MM4 x DD6) F_1 hybrid also produced strong clonal expansion and AFC production of comparable level to lines expressing the IgG membrane tail.

How could this pattern of reactivity be reconciled with differences in IgM and IgD isotype signalling?

Firstly, it is possible that the increased response made by the IgMδE lines compared to the IgM-only line reflects altered signalling through the BCR complex, as the transgenic constructs used to make the IgMδE lines contain the 27-amino acid extracellular spacer of IgD. This small section was shown *in vitro* to confer an IgD-like glycosylation pattern to Igα associated with the BCR (Pogue, 1994). It may be that this change in glycosylation pattern confers a more "IgD-like" signal upon antigen engagement of the BCR.

Secondly, there is a previous precedent for differences in the responsiveness of IgM and IgD transgenic B cells to antigen in this system. A similar order of reactivity to antigen

in vivo was seen amongst MM7, MD4 and DD6 anti-HEL transgenic B cells seeded into irradiated recipients immunised with HEL-SRBC (Brink, 1992). In that case, the IgM-only (MM7, sister line to MM4) cells produced ~10-fold lower serum IgM^a compared to the MD4 cells. In addition, the MD4 cells produced ~10-fold lower serum IgM^a compared to the level of serum IgD^a made by DD6 cells.

In the current study there are several possible reasons for the strong clonal expansion and AFC response made by the DD6 and MM4xDD6 lines. Firstly, the DD6 and MM4xDD6 lines express similar, elevated levels of anti-HEL IgD^a compared to the MM4 or MD4 lines (Figure 6-1, part C). Ignoring isotype, it is conceivable that the simple ~2-fold higher expression levels of antigen-specific BCR in the DD6 and MM4xDD6 lines is responsible for their stronger response to antigen. This seems unlikely, however, as the GG4 line makes very robust responses to antigen, yet has ~5-fold lower anti-HEL receptor levels compared to the MM4 line (Pogue, 1996) (also see Figure 5-4). If isotype is taken into account, it could be argued that the DD6 and MM4xDD6 lines respond well to antigen because of unusually high anti-HEL IgD BCR expression when compared to the MM4, MD4 or MδE lines. The signalling through the IgD BCR complex on those cells would then be hypothesised to protect from apoptosis during the process of clonal expansion, in a similar way to the action of the IgG membrane tail.

Despite these speculations, it is difficult to provide a physiological interpretation for the increased reactivity of lines expressing high levels of anti-HEL IgD. Firstly, the dysregulated expression of IgD from a transgene during bone marrow development and the secretion of IgD by plasma cells does not mirror the tight regulation of IgD expression during B cell development in either normal or MD4 mice (Brink, 1992). Secondly, functional differences between IgD and IgM *in vivo* have been largely discredited, despite early suggestions that signalling through IgD was responsible for the switch from tolerance to immune responsiveness that accompanies the immature to mature B cell transition (Carsetti, 1993). Subsequent experiments showed that IgM-only transgenic B cells develop into mature B cells normally and make normal responses to antigen in the absence of IgD (Brink, 1992), and the only defects noticed in IgM knockout mice were slightly delayed responses to some viral antigens (Lutz, 1998). These experiments suggest that the maturation state of the B cell seems more important than isotype expression in determining the response the B cell makes to antigen (Norvell, 1996).

This raises the possibility that B cells in these various transgenic lines are somehow "stuck" in different developmental stages. In support of this, the MM7 line (generated from the same construct as MM4) has a greater fraction of HSA^{hi} $B220^{lo}$ phenotype splenic B cells compared to the MD4 line, possibly indicating a greater fraction of immature

phenotype B cells in the spleen (Brink, 1992). Despite this, all of the lines tested in this study make phenotypically normal mature follicular B cells, which means a simple explanation for differences in immune responsiveness in terms of B cell maturation state is unlikely.

To summarise, the interesting observation of variable immune responsiveness amongst MM4, MD4, MδE1-2, DD6 and MM4xDD6 lines hints that varying levels of IgD expression may play a role in naïve anti-HEL transgenic B cell responses to antigen *in vivo*. Whether this is due to the simple variation in antigen receptor levels, some consequence of acute antigen stimulation *via* IgD isotype receptors, or a reflection of developmental differences in peripheral B cells between the lines remains to be determined.

Section 6.5. Chapter summary

This chapter has dealt with the important issue of line to line variation as a source of differential responses to antigen by the variety of transgenic lines reported in previous chapters. This is a critical point that is central to the interpretation of the results presented in this thesis, as this experimental system relies on comparisons between B cells that must be as closely matched as possible apart from the expression of different BCR isotypes. If this condition can not be met or be shown experimentally, then this system cannot be used to isolate the role of BCR isotype in B cell responses to antigen.

There is clear evidence for differences between the various lines used in this study which are not connected with BCR isotype. As with any panel of conventional Ig transgenic mice, line to line variation due to copy number and integration site effects must be taken into account (Goodnow, 1992). Variation in the switch region sequences of the transgenic constructs used to make this panel of mice (Table 6-1) must also be addressed.

In addition, differences in B cell development exist between the lines that may be connnected to the expression of different BCR isotypes. For example, edited B cells that have lost HEL-binding accumulate in the periphery of IgG and IgM/G transgenic lines (Pogue, 1996; Pogue, 2000). The degree of editing varies with transgene copy number which may reflect the strength of signalling by the IgG membrane tail, leading to developmental arrest and editing. This complicates the analysis, as edited peripheral B cells may retain some low-level expression of HEL-binding BCR, and peripheral HEL-binding B cells in these lines might be altered by editing events that do not take place to the same degree in the other transgenic lines.

This thesis has also extended the analysis of the differences in peripheral B cells between these lines, by showing that there are large variations in the fraction of peripheral HEL-binding cells that have a marginal zone versus follicular surface phenotype. This may be connected to BCR expression, but whatever the cause, it means that adoptive transfers with unfractionated spleen cells transfer different proportions of marginal zone and follicular B cells.

The results presented in this chapter rule out several trivial explanations for the responses of the various Ig transgenic B cells to antigen *in vivo*. Line to line variation is excluded as a source of differential responses to antigen, as consistent responses are made by the three independent MG lines and the two independent MδE lines. In addition, construct switch region type has no bearing on the magnitude of the response. Lines bearing identical transgene switch regions but differing in surface Ig isotype (e.g. MG lines and MδE lines) make very different responses to antigen, while lines bearing different transgene switch regions but sharing the IgG membrane tail make similar responses to antigen (e.g. GG4 and MG lines). In addition, the presence of an intact μ switch region is not responsible for the reduced net clonal expansion of the MM4 line, as the MM4xDD6 line, while retaining that switch region, still makes a very robust response to antigen. It is important to note that transcription and translation of the IgM transgene remains high in the MM4xDD6 B cells (Bell, S. and Goodnow, C.C., unpublished observations).

Most importantly, phenotypic heterogeneity within the peripheral B cell compartments can be excluded as an explanation for differential responses to antigen between the various transgenic lines. Transfer experiments using transgenic B cells from RAG1-deficient spleen and lymph node rule out the possibility that the edited non-HEL-binding B cells that accumulate within the spleen of IgG and IgM/G transgenic lines carry low levels of HEL-binding receptors or are involved in immune responses upon adoptive transfer. Also, transfer experiments using homogeneous matched immature bone marrow B cells or mature lymph node B cells demonstrate that phenotypic variation or selection in the peripheral splenic marginal zone and follicular subsets of donor mice is not necessary for differential responses to antigen. The transfer experiment using sorted splenic MZ and FO phenotype B cells, while providing variable, inconclusive results, did not indicate large differences in reactivity between the two subsets. In each case IgG transgenic MZ or FO phenotype cells produced more AFCs on adoptive transfer compared to IgM transgenic MZ or FO phenotype cells.

The finding that variations in the fraction of marginal zone and follicular HEL-binding peripheral B cell subsets between transgenic lines is not necessary to retain differential response to antigen is a surprising result, given the special qualities normally

attributed to marginal zone B cells. For example, they have been reported to have a pre-activated phenotype as well as to rapidly migrate and differentiate upon antigen encounter (Liu, 1991; Liu, 1995; Arpin, 1997; Oliver, 1997a; Oliver, 1999; Martin, 2001). How can the findings presented in this thesis that a marginal zone phenotype does not necessarily correlate with antigen reactivity be reconciled with these observations? Firstly it may be inappropriate to compare many of these reports with the current study, given the heterogeneous makeup of the marginal zone B cell compartment in normal mice. Model systems demonstrating the rapid activation of memory B cells (Liu, 1991) or "polyreactive" B cells (Martin, 2001) may be measuring primarily the effects of prior antigen priming coupled with localisation to a key antigen-draining site. A significant population of "naïve" B cells that accumulate in the marginal zone of normal rats show no signs of somatic mutation (Dammers, 1999a; Dammers, 2000), and yet studies of the rate of replacement of the marginal zone pool and V_H gene useage suggest that selective forces among these cells are strong. By contrast, Ig transgenic mice that contain relatively high proportions of monoclonal B cells must disrupt the selective pressures existing in normal animals by decreasing the competition for entry into the marginal zone pool. The data presented in this thesis suggests that the level of clonal competition is one important determinant of the relative fraction of anti-HEL Ig transgenic cells that adopt a marginal zone phenotype (see Figure 5-5). It may be that the "space" normally reserved in the marginal zone for selected B cell clones can be filled by normally excluded clones in Ig transgenic mice as a default pathway, leading to marginal zone phenotype B cells that lack many of the functional properties derived from selection and antigen contact that are normally associated with those cells.

One interesting factor contributing to reactivity to antigen may be variations in the expression of IgD amongst several anti-HEL transgenic lines. While lines expressing normally regulated IgM or IgD (MM4, MD4, MδE1, MδE2) make >10-fold poorer responses to antigen compared to lines expressing the IgG membrane tail, lines carrying a dysregulated IgD transgene (DD6, MM4xDD6) make very robust responses. An explanation of the impact of higher IgD expression on B cell responses to antigen is difficult to reconcile with previous data, and is beyond the scope of this thesis to address.

To conclude, the data here exclude trivial explanations of line to line variation in the pattern of immune reactivity seen amongst the extensive panel of anti-HEL Ig transgenic mice used in this study. More surprisingly, data presented here shows that differences in the stage of B cell development or variations in marginal zone versus follicular phenotype amongst antigen-specific cells do not contribute to the observed differences in reactivity to antigen *in vivo*. Thus, expression of the IgG membrane tail on B cells that are

developmentally matched in all other respects allows more robust responses to antigen compared to B cells expressing the naïve IgM BCR isotype.

Section 6.6. Future work

In the future it would be useful to repeat the active sorting of marginal zone and follicular B cells from the IgM transgenic line and lines expressing the IgG membrane tail. By transferring a greater number of cells (at least 10^6) it may be possible to overcome the variable responses seen in this study. A comparison of the effects of CD21 ligation during sorting and adoptive transfer should also be carried out, to determine whether sorting MZ B cells using this parameter is overtly stimulatory. Alternative, potentially less stimulatory sorting parameters exist, such as CD1d (Roark, 1998).

Chapter 7. Biochemical analysis of early signalling by IgG isotype receptors

Section 7.1. Introduction

The previous chapters have established that the expression of the IgG membrane tail confers enhanced reactivity to antigen during an immune response *in vivo*. The membrane tail acts by protecting B cells from loss during the process of clonal expansion, although the biochemical mechanism behind this protective effect is currently unknown.

This short chapter describes preliminary biochemistry experiments designed to test whether there are early BCR signalling differences between IgG, IgM/G and IgM isotype anti-HEL transgenic B cells. This is an important line of investigation that aims to combine the cellular observations of heightened reactivity to antigen during an immune response with isotype-specific biochemical pathways operating in B cells bearing the IgG membrane tail.

Several previous lines of evidence have suggested that BCR signalling *via* anti-HEL receptors bearing the membrane tail of IgG is qualitatively different compared to lines expressing combinations of IgM and IgD. For example, despite expressing equivalent levels of transgenic receptors on pro-B bone marrow lineage cells compared to transgenic lines expressing IgM and IgD, lines expressing the IgG membrane tail accumulate "edited" B cells in the periphery that have lost the ability to bind HEL (Pogue, 2000). As well, peripheral HEL-binding B cells in the IgG and IgM/G lines express lower receptor levels (80 % and 40 % lower respectively) compared to the IgM-only line (Pogue, 1996). This is reminiscent of the receptor modulation made by MD4 anti-HEL B cells deficient in SHP-1 (Cyster, 1995), CD22 (Cornall, 1998), Lyn kinase (Cornall, 1998) or overexpressing CD19 (Inaoki, 1997), and suggests that IgG membrane tail signalling may be of a different quality, leading to compensatory mechanisms such as receptor editing and receptor down-modulation.

In vitro experiments with IgG, IgM/G and IgM transgenic anti-HEL lines have focussed on early signal transduction events surrounding BCR stimulation, using cross-linking antibodies or the cognate antigen HEL in either monomeric or multimeric form (Pogue, 1996). Some signalling pathways were more active in transgenic B cells expressing the IgG membrane tail. For example, elevated basal intracellular calcium was observed in IgG and IgM/G mature transgenic B cells compared to the IgM transgenic B cells. In addition, the IgG and IgM/G transgenic B cells made a more robust calcium flux to multimeric HEL-dextran and anti-kappa antibodies. However, some signalling pathways were less active in transgenic B cells expressing the IgG membrane tail. For example, the induction of tyrosine phosphorylation of intracellular cytoplasmic proteins was poor in IgG

and IgM/G transgenic B cells by either HEL or anti-kappa stimulation. These *in vitro* observations suggested that the IgG isotype BCR coupled more efficiently to some downstream signalling pathways, as the poor induction of tyrosine phosphorylation was still accompanied by a robust calcium response. These observations also suggested that signalling *via* the IgG or IgM isotypes in this system diverge very soon after antigen receptor engagement.

The movement of both B and T cell antigen receptors into detergent-insoluble, cytoskeleton-associated membrane microdomains (lipid rafts) has been proposed as a very early step required for proper antigen receptor signalling in mature, naïve lymphocytes (Cheng, 1999; Viola, 1999; Weintraub, 2000; Cherukuri, 2001). It was reasoned that IgG and IgM/G BCR receptors might signal more efficiently due to constitutive association with lipid rafts that are rich in positive signalling molecules such as Src family kinases and Syk, while excluding negative regulators of signalling such as the protein tyrosine phosphatase CD45 (Weintraub, 2000; Cherukuri, 2001). In this study the partitioning of IgG, IgM/G and IgM BCRs into cytoskeleton-associated lipid rafts was investigated to determine whether the IgG and IgM/G isotype receptors were arranged differently at the cell surface.

Section 7.2. IgG transgenic B cells show a different pattern of early activation compared to IgM transgenic B cells

All of the experiments described in this chapter were performed with primary splenic B cells from Ig transgenic lines on the RAG1$^{-/-}$ background, allowing the isolation of a pure population of HEL-binding B cells.

In agreement with the findings of Pogue (Pogue, 1996), the induction of tyrosine phosphorylation on bulk cytoplasmic proteins was consistently lower upon antigen receptor stimulation in IgG transgenic B cells compared to IgM transgenic B cells. A representative experiment is shown in Figure 7-1 where detergent lysates and the detergent-insoluble fraction from resting or HEL-stimulated IgG:RAG1$^{-/-}$ and IgM:RAG1$^{-/-}$ primary B cells were analysed by Western Blot. Panel A shows the blot probed for anti-phosphotyrosine. While there was little induced tyrosine phosphorylation in the IgG detergent-soluble fraction, the IgG detergent-insoluble fraction contained several proteins that became phosphorylated on stimulation. The opposite was seen in the IgM samples, as there were several proteins that were inducibly phosphorylated in the detergent-soluble fraction upon

stimulation, but there were few phosphorylation changes in proteins associated with the IgM detergent-insoluble pellet.

An interesting observation was made with respect to the isoforms of Igαβ that associate with the IgG or IgM isotype antigen receptors. Panel B shows the blot in panel A re-probed with anti-sera against Lyn, Igα and Igβ. There are distinct molecular mass isoforms of Igα associated with the detergent soluble and detergent-insoluble fractions in both IgM and IgG transgenic B cells. This heterogeneity in Igα molecular mass has been observed previously, for example between IgD and IgM-associated molecules, and reflects differential N-glycosylation (Campbell, 1991; Pogue, 1994). The IgG transgenic B cells lack the lower ~30 kDa molecular mass isoform indicated by the star. In addition, there is a mobility shift of the intermediate Igα species between the detergent-soluble (~32-33 kDa) and insoluble (~ 34 kDa) fractions in both IgG and IgM transgenic cells. It is tempting to speculate that this mobility shift reflects phosphorylation and that a large fraction of the active, phosphorylated IgM and IgG receptors are present in the detergent-insoluble material.

Section 7.3. The IgG BCR may constitutively associate with lipid rafts

Cytoskeleton-associated membrane rafts were investigated next, as the movement of the BCR into these membrane domains upon receptor cross-linking is one of the earliest events in antigen receptor signalling in B cells, and is a step that is uncoupled in B cell anergy (Weintraub, 2000). An isolation procedure using high salt washes of detergent-insoluble material that has been previously validated in primary naïve and anergic B cells (Weintraub, 2000) was used to test whether the IgG isotype BCR associates more readily with cytoskeleton-associated membrane rafts, either constitutively or after *in vitro* stimulation. This technique was first applied to the association of the activated TCR with the T cell cytoskeleton (Marano, 1997) and the method involves extracting cells with non-ionic detergent in a buffer that stabilises the cytoskeleton. Most of the plasma membrane is dissolved into the supernatant by the detergent, but a small fraction of the membrane remains detergent insoluble and attached to the cytoskeleton. When the NaCl concentration is increased, attached membrane fragments are released into the supernatant. Figure 7-2 shows results from RAG1-deficient IgG, IgM/G and IgM transgenic B cells that were stimulated *in vitro* with HEL, then low salt detergent and high salt detergent fractions were prepared under non-reducing conditions and analysed by Western blot. The BCR complex was detected by probing for kappa light chain under non-reducing conditions, which allowed IgG, IgM and IgM/G isotypes to be distinguished on the basis of molecular mass.

These data are in accordance with previous studies using the MD4 anti-HEL line (Weintraub, 2000), and show the movement of the IgM isotype BCR from MM4:RAG1$^{-/-}$ B cells into the salt extractable cytoskeleton-associated membrane raft fraction after stimulation. This has been a consistent observation in four out of four experiments performed using this system. These data also show the constitutive presence of the IgG isotype BCR in the membrane raft fraction from GG4:RAG1$^{-/-}$ B cells, with little enrichment after stimulation. The same pattern has also been observed with IgM/G isotype MG2:RAG1$^{-/-}$ B cells. However, for both IgG and IgM/G transgenic B cells the constitutive association with membrane rafts was not consistent, being observed in only two out of three experiments.

Figure 7-1. TX-100 lysis of stimulated IgG:RAG1$^{-/-}$ and IgM:RAG1$^{-/-}$ transgenic B cells showing differences in tyrosine phosphorylation and Igαβ isoforms

Results from a representative experiment in which 10^7 IgG:RAG1$^{-/-}$ or 10^7 IgM:RAG1$^{-/-}$ transgenic B cells were stimulated ± HEL (10 μg/ml) for 3 minutes at 37 °C. After stimulation, cells were lysed in buffer containing 1 % (v/v) TX-100 with protease and phosphatase inhibitors (see materials and methods Section 2.12). 1.5 x 10^6 cell equivalents of soluble lysate and 0.75 x 10^6 cell equivalents extracted from the insoluble lysate using sample buffer were run on a 10 % reducing polyacrylamide gel and transferred to a PVDF membrane for Western blotting.

A. Western blot probed with anti-phosphotyrosine mAb (clone 4G10) followed by goat anti-mouse IgG F(ab')$_2$ fragments conjugated to horse radish peroxidase (ICN). Molecular mass markers are shown at the left by bars.

B. The same blot as in (A), re-probed with a mixture of anti-Lyn, anti-Igα and anti-Igβ antibodies (all rabbit anti-mouse polyclonal sera), followed by goat anti-rabbit HRP (Zymed). The position of the p53/p56 Lyn isoforms as well as Igβ and Igα isoforms are shown. Stars indicate the low molecular mass ~30 kDa Igα isoform present in the IgM samples and the intermediate molecular mass ~32-33 kDa Igα isoform that shows a mobility shift in both samples between the two cellular fractions. Molecular mass markers are shown at the left by bars. The high background on this blot may reflect residual HRP activity from the blot in (A), as is was not stripped before re-probing. However, this has no stopped the unequivocal identification of the target proteins Lyn, Igα and Igβ.

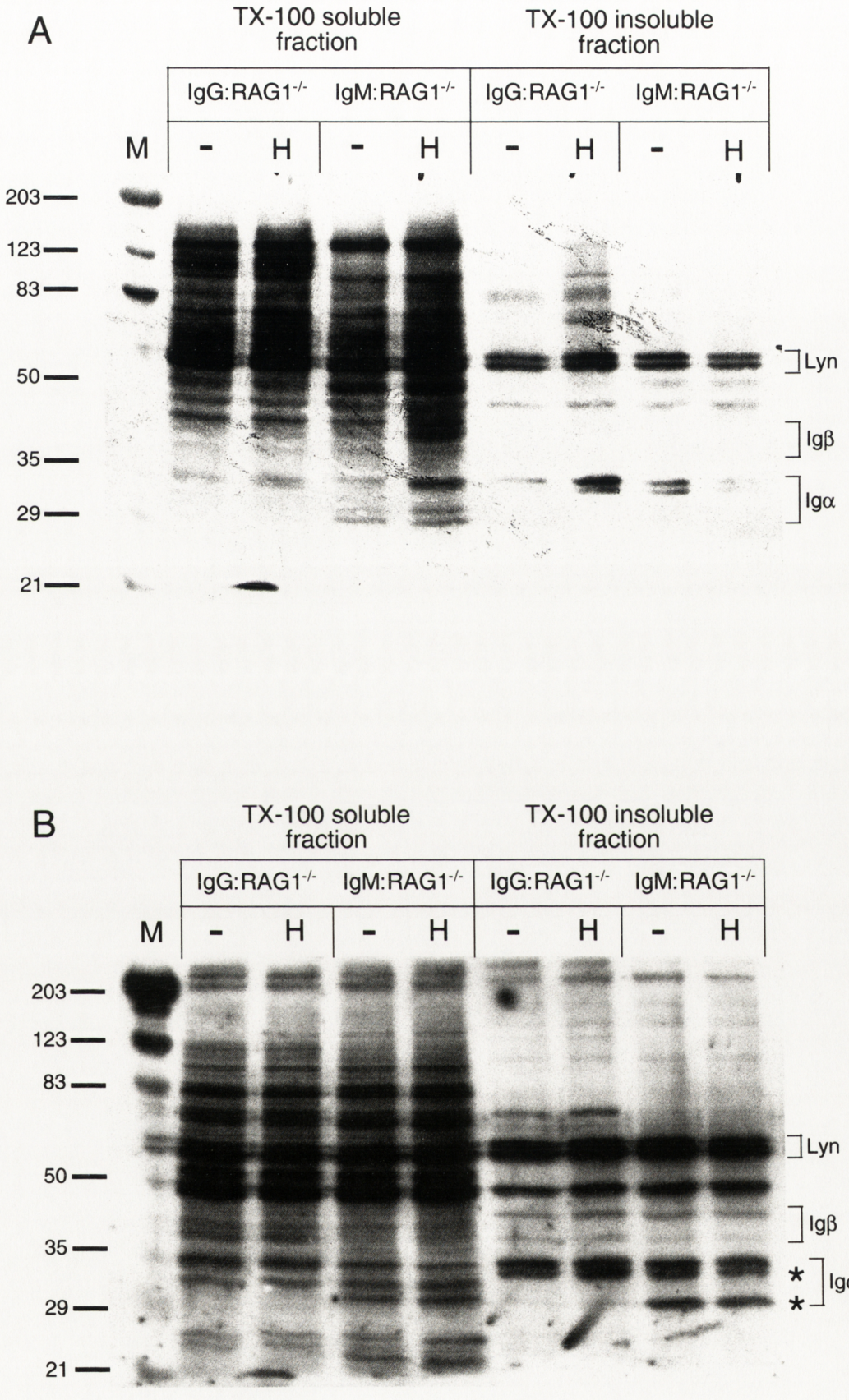
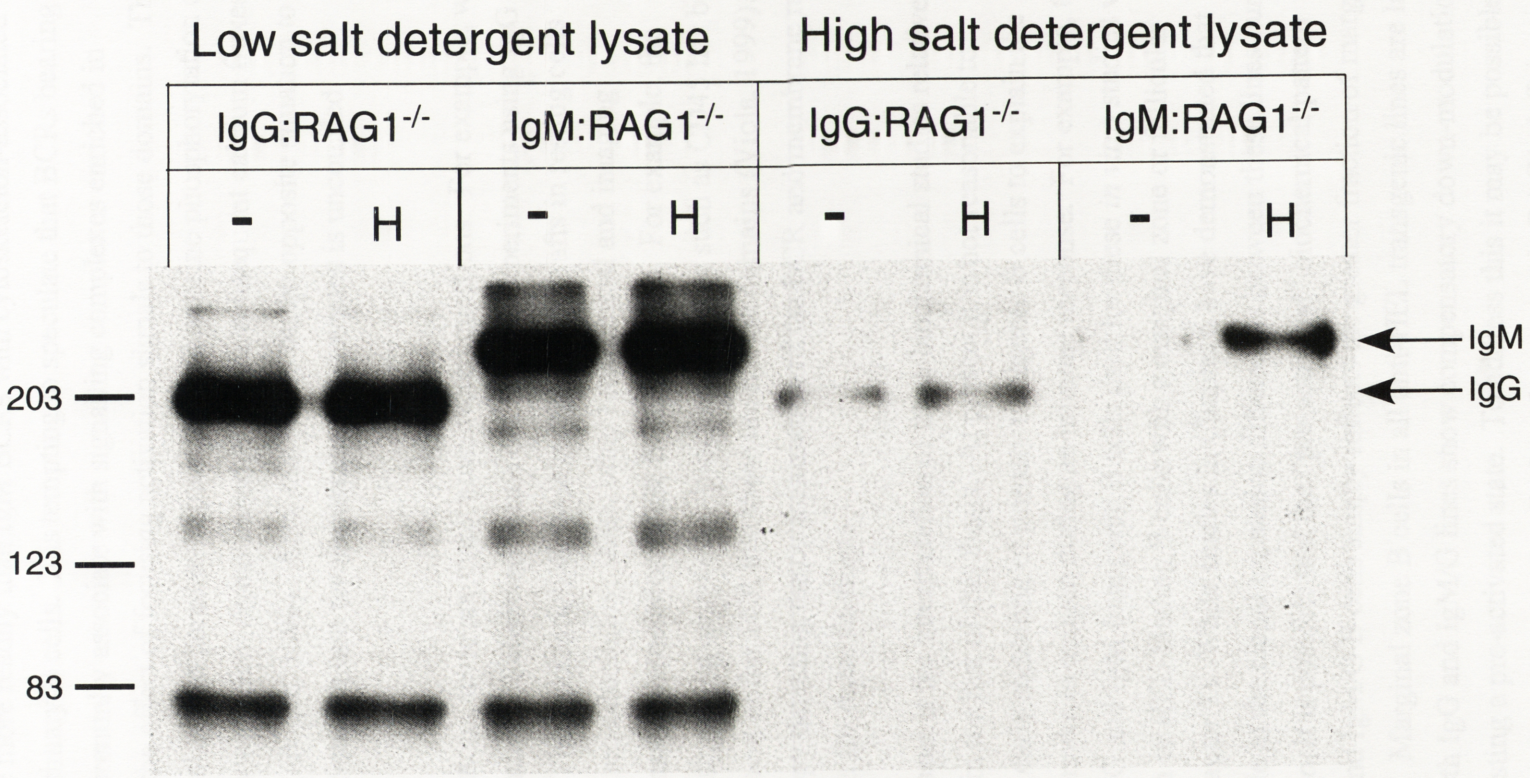

A
TX-100 soluble fraction
TX-100 insoluble fraction
IgG:RAG1-/-
IgM:RAG1-/-
IgG:RAG1-/-
IgM:RAG1-/-
M
-
H
-
H
-
H
-
H
203
123
83
50
35
29
21
Lyn
Igβ
Igα
B
TX-100 soluble fraction
TX-100 insoluble fraction
IgG:RAG1-/-
IgM:RAG1-/-
IgG:RAG1-/-
IgM:RAG1-/-
M
-
H
-
H
-
H
-
H
203
123
83
50
35
29
21
Lyn
Igβ
Igα
*
*

Figure 7-2. The IgG isotype BCR may constitutively associate with a salt-extractable cell compartment corresponding to cytoskeleton-associated membrane rafts

10^7 IgG:RAG1$^{-/-}$ or IgM:RAG1$^{-/-}$ transgenic B cells were stimulated ± HEL (10 μg/ml) for 3 minutes at 37 °C in the same experiment outlined in Figure 7-1. These samples were then lysed in low salt detergent containing 0.1 % Triton X-100 as well as protease and phosphatase inhibitors (see materials and methods). The detergent soluble "low salt" fraction corresponding to cytoplasmic material was collected. After washing, the detergent-insoluble pellet was resuspended in high salt CSB buffer to collect the high salt-extractable fraction enriched for cytoskeleton-associated membrane rafts. All samples were boiled in non-reducing sample buffer. 10^6 cell equivalents of low salt and $4x10^6$ cell equivalents of high salt fractions were run under non-reducing conditions on 7.5 % polyacrylamide gels. Western blotting to PVDF membranes was performed as described in part 2.12 (b) of the materials and methods.

The Western blot was probed using biotinylated goat anti-mouse kappa light chain (Southern Biotechnology), followed by streptavidin/biotin-HRP conjugate (Amersham). The position of the IgG and IgM BCRs are indicated by the arrows. Molecular mass standards are indicated at the left by bars.


Low salt detergent lysate
High salt detergent lysate
IgG:RAG1-/-
IgM:RAG1-/-
IgG:RAG1-/-
IgM:RAG1-/-
-
H
-
H
-
H
-
H
203
123
83
IgM
IgG

Section 7.4. Chapter summary and future work

This short chapter has outlined preliminary biochemical evidence that IgG and IgM/G isotype BCRs may associate more readily than IgM BCRs with cytoskeleton-associated membrane rafts in resting primary B cells. It is tempting to speculate that BCRs bearing the IgG membrane tail preferentially associate with signalling complexes enriched in membrane rafts, which has the effect of focusing activation signals to those domains. This may explain the previously observed paradox of poor induced tyrosine phosphorylation of most cytoplasmic proteins but efficient signal transduction for subsequent calcium fluxes upon receptor cross-linking (Pogue, 1996). Also, this would be the opposite situation to B cell anergy where BCR partitioning into the membrane raft domains is uncoupled (Weintraub, 2000).

Despite these observations, there are many unanswered questions. For example, why was constitutive raft association not observed in one out of three experiments using IgG or IgM/G transgenic B cells? The association of these receptors with rafts in resting cells needs to be repeated to test reproducibility. Also, other biochemical and imaging techniques are available to assess association of the BCR with rafts. For example, the ability of rafts to sequester signalling complexes from phosphatases such as CD45 has been used to demonstrate movement of the TCR into these membrane domains (Viola, 1999). As well, confocal microscopy detecting the co-localisation of the BCR and membrane rafts could provide corroboration for these findings.

Another significant issue is the interpretation of these biochemical studies relative to the cellular data presented in the rest of this thesis. At present it is not reasonable to extrapolate findings on the early signalling of primary transgenic B cells to explain the differential responses they make to antigen during an immune response. For example, the RAG1-deficient IgG, IgM/G and IgM transgenic B cells used for these *in vitro* studies vary considerably in the fraction of HEL-binding B cells with a marginal zone or follicular surface phenotype (see Chapter 5). While studies in Chapter 6 have demonstrated that these variations play no role in the clonal expansion differences between these lines during an immune response *in vivo*, it is possible that the "pre-activated" biochemical pattern observed here in the IgG and IgM/G B cells simply reflects the greater fraction of marginal zone B cells in those lines. Marginal zone B cells in all anti-HEL transgenic lines are larger (see Figure 5-2), and in both IgG and IgM/G lines show compensatory down-modulation of surface BCR levels, suggesting a pre-activated state. To address this it may be possible to perform these experiments on lymph node B cells from Ig transgenic RAG1-deficient mice, which show a uniform cell surface phenotype (Figure 6-3) and are more closely matched.

Also, a biochemical explanation for the cell biology of this system must explain how differences between cells bearing the IgG membrane tail and those bearing IgM are not observed during the initial activation into cell cycle, but manifest between days 3 and 5 of an immune response *in vivo* (described in Chapter 4).

There are two possible scenarios by which the IgG membrane tail could mediate this effect. Firstly, signalling differences between IgG and IgM isotype receptors might occur throughout cell activation and cell division, but only affect cell biology in the system at later timepoints. In this case the IgG membrane tail might alter the relative balance of pro- and anti-apoptotic proteins in the cell, in a similar way to CD28 (Sperling, 1996; Gudmundsdottir, 1999) signalling during T cell clonal expansion. This may only affect the survival of cells expressing the IgG membrane tail at later timepoints, perhaps when antigen or T cell help becomes limiting, or when a cross-linking form of antigen is presented to the B cell. The relative levels of pro- and anti-apoptotic signalling pathways could be studied biochemically directly *ex vivo*, either during acute stimulation or after short-term culture.

Secondly, the relevant signalling differences may only be present at later timepoints. Perhaps differential signalling by the IgG membrane domain is only induced after some change in the immune response, such as antigen becoming limiting or the only available antigen being held in a highly cross-linking manner as immune complex. If this is the case, then the most useful approach may be to sort Ig transgenic cells from ongoing immune responses at relevant timepoints in order to conduct micro-array experiments. Once candidate genes have been identified that could be involved in the survival effect of the IgG membrane tail, a clear picture of signalling pathways affected by expression of the IgG membrane tail should emerge.

Chapter 8. General Discussion

Section 8.1. Introductory comments

This thesis has investigated the role of BCR isotype in B cell responses to T-dependent antigens. The experimental system of naïve Ig transgenic B cells bearing defined BCR isotypes was able to remove the other variables of frequency, priming status, location, surface phenotype and lifespan that often accompany isotype switching. In this way the contribution of BCR isotype to B cell responses could be studied in isolation.

This discussion will briefly summarise the findings of this study. Three key questions will then be addressed. (a) How does the current study add to the literature concerning the functional role of isotype-switched antigen receptors? (b) What are the possible biochemical mechanisms behind the protective action of the IgG membrane tail? (c) What is the importance of isotype-switched BCR expression for the features of the memory response? Finally, future work will be outlined to test some of the predictions made by this study.

Section 8.2. The role of BCR isotype in B cell responses to antigen

8.2 (a) Summary of the major findings:

The major finding of this study is that anti-HEL transgenic B cells expressing the unique membrane tail of IgG_1 make at least a 10-fold greater clonal expansion and AFC response compared to transgenic B cells expressing various combinations of IgM and IgD during a primary TD response *in vivo*. This is strong evidence for a distinct, functional role for BCR isotype during an immune response *in vivo*.

The increased clonal expansion of B cells bearing the IgG membrane tail is not due to greater initial activation, as an equivalent proportion of IgM, IgM/G and IgG transgenic cells enter cell division cycle on day 2 of this model immune response.

The IgG, IgM/G and IgM transgenic B cells appear to have similar rates of cell division, as assessed by CFSE dilution on day 3 and day 5 after immunisation and transfer, which reveals the mode of action of the IgG membrane tail. The IgG membrane tail acts to protect transgenic B cells from loss (most likely by cell death) during the process of clonal expansion.

This is consistent with histological studies, where transgenic B cells expressing or lacking the IgG membrane tail are found in similar extrafollicular sites near the junctional zones of the spleen on days 3 and 4 after transfer. By day 5, however, a large accumulation of syndecan-binding transgenic B cells in the junctional zones is apparent in mice bearing the IgG membrane tail, while few of the IgM transgenic B cells make this transition.

Transgenic B cells in this system, regardless of isotype, apparently modulate BCR levels strongly on day 4 after immunisation and transfer during a primary response to HEL in the presence of TCR transgenic helpers. This is hypothesised to be the result of contact with a highly cross-linking form of HEL, and only transgenic cells expressing the IgG membrane tail survive the encounter to make the transition to extrafollicular plasma cells with the recovery of surface Ig expression. Most transgenic B cells expressing IgM or IgD receptors do not survive.

Cell death induced by FasL expression on transferred transgenic helper T cells is not responsible for the loss of transgenic cells expressing IgM and IgD isotype receptors, although a role for FasL expressed by endogenous recipient T cells or dendritic cells is still possible. The role of the Fas/FasL death pathway in limiting IgM transgenic cell clonal expansion could be more thoroughly ruled out by transfers into $FasL^{gld}$ recipient mice. Apoptotic cell death remains the hypothesised pathway of IgM transgenic cell loss, and possibly involves encounter with an immuno-modulatory form of HEL as discussed above.

Two other interesting preliminary findings have also been made:

Firstly, the expression of IgD along with IgM seems to decrease the sensitivity of transgenic B cells to cell loss. The MD4 line (IgM and IgD), for example, consistently made a more robust clonal expansion and AFC response than the MM4 line (IgM-only). The nature of the protective effect is unknown and is difficult to reconcile with previous data that has shown no functional differences between IgM and IgD (Roes, 1991; Brink, 1992; Roes, 1993; Lutz, 1998). The most extreme example of this effect is in lines carrying an IgD transgene that is independently expressed (DD6 and MM4xDD6). These lines made responses comparable to lines expressing the IgG membrane tail. The strong *in vivo* anti-HEL response of the IgD-only line is certainly consistent with previous observations of reactivity between IgM-only, IgM/IgD and IgD-only lines to HEL-SRBC (Brink, 1992). Several differences, including a combination of higher surface receptor expression and the dysregulation of normal IgD expression, especially with respect to early expression in the bone marrow as well as the development of IgD-secreting plasma cells (Brink, 1992), make results using these lines difficult to interpret.

Secondly, transgenic B cells expressing the IgG membrane tail and transgenic B cells expressing IgM can both participate in both follicular and germinal centre pathways during an immune response. After seeding into short-term primed recipients, both IgM and IgM/G transgenic B cells participated in follicular clonal expansion, and by day 5 some transgenic B cells of each type had entered germinal centres. Interestingly, this method of priming completely abolished the dominant extrafollicular response seen in the anti-lysozyme response in unprimed mice in the presence of TCR transgenic helpers, providing a useful system for the future to dissect the possible role of helper T cells in driving germinal centre versus extrafollicular focus responses.

8.2 (b) Validation of the experimental model

The experiments in this thesis compared many different anti-HEL Ig transgenic lines expressing different isotype BCRs. In order to make valid conclusions about the role of BCR isotype in B cell responses it was important to ensure that line-to-line construct and phenotypic differences would not complicate the analysis.

Several issues needed to be addressed. The transgenic lines were created using constructs that differ in switch region sequences and which potentially integrated in a variety of sites and might affect line-to-line comparison. Also, previously documented differences in splenic B cell phenotype existed between the three principal lines (GG4, MG2 and MM4) used in this study. This included varying expression of CD21 and BCR on mature B cells, as well as varying degrees of receptor editing away from the transgene specificity (Pogue, 1996; Pogue, 2000). In fact, this thesis has extended the analysis of these differences, by showing that the increased fraction of $CD21^{hi}$ HEL-binding cells in the IgG and IgM/G lines compared to the IgM-only line is due to a greater fraction of HEL-binding cells with a marginal zone location and $CD21^{hi}$ $CD23^{-}$ surface phenotype. When many transgenic lines were analysed there was wide variation in the fraction of marginal zone and follicular phenotype HEL-binding cells, probably due to a complicated combination of clonal competition for marginal zone entry as well as BCR isotype effects.

The issue of line-to-line variation was discounted as an explanation for differential responses of transgenic B cells to antigen, as lines made with the same construct gave consistent responses, and variations in transgene switch region did not correlate with variations in reactivity to antigen. Even though an extensive panel of transgenic lines was used, heightened reactivity to antigen *in vivo* only correlated with the expression of the IgG membrane tail (or a dysregulated form of IgD). In a similar way, the effect of edited B cells in IgG and IgM/G lines that no longer bound antigen was discounted, as transgenic B cells from $RAG1^{-/-}$ or $RAG1^{+/+}$ mice had the same pattern of reactivity to antigen *in vivo*.

Surprisingly, the variable proportion of marginal zone and follicular phenotype HEL-binding B cells in each line was also discounted as a factor in differential responses to antigen. Firstly, some lines with a relatively low fraction of HEL-binding marginal zone phenotype cells still make a similar clonal expansion response compared to lines with many HEL-binding marginal zone phenotype cells. Secondly, two experiments using matched immature bone marrow or mature lymph node B cells recapitulated the differential responses to antigen, even though there was no evidence of cell surface phenotypic heterogeneity in the B cells used.

The validation of this experimental system was crucial to the conclusions that can be drawn from it. Despite every effort to test the effect of line-to-line heterogeneity as a cause of the different responses to antigen, BCR isotype is the only factor that predicts the magnitude of the transgenic B cell response to antigen. The IgG membrane tail consistently protects B cells from loss during a primary immune response *in vivo* and allows a greater net clonal expansion compared to B cells expressing normally-regulated combinations of IgM and IgD.

8.2 (c) Integration of these findings with other studies of switched isotype BCR function

The study outlined in this thesis provides a potential mechanism to bring together many previous observations about the role of isotype-switched B cells during an immune response. This section will review how this study encompasses previous work and will highlight several uncertainties that still need to be addressed.

A series of early papers concerned with the relationship of BCR isotype and B cell function showed a strong correlation between memory B cells and the expression of isotype-switched receptors, in particular IgG. It was shown that depletion of IgG^+ cells from primed lymphocyte mixtures, either by radioactive antigen (Coffman, 1977) or complement-mediated lysis (Yuan, 1977) decreased the adoptively transferrable memory response. In addition, later studies using primed, antigen-specific B cells isolated by a rosetting method (Yefenof, 1986) or by cell sorting (Hayakawa, 1987) showed that *in vitro* memory responses by these cells were mostly due to IgG^+ B cells. Interestingly, the study of Yefenof and colleagues (Yefenof, 1986) demonstrated, using limiting dilution assays, that IgG^+ memory cells had a much larger "clone size" of plaque-forming cells compared to the IgG^- memory cell fraction. From the results presented in this thesis, it is tempting to speculate that the dominance of IgG^+ B cells in driving memory responses *in vivo* or during culture *in vitro* is due to IgG membrane tail expression, which allows a robust clonal burst by protecting B cells from loss during clonal expansion.

The early work presented above provided only correlative evidence that expression of IgG was a component of the heightened responses of memory B cells. The contribution of BCR isotype was masked in those studies by the other changes that accompany memory cell formation. They were useful in defining the phenotypes of memory B cells and showing that memory B cells had decreased activation requirements compare to naïve B cells, but gave no way of assessing the contribution of IgG expression in isolation. In fact, aside from *in vitro* biochemical experiments into the mechanisms of isotype-specific BCR surface expression (Venkitaraman, 1991; Weiser, 1994; Knight, 1997), BCR signalling (Leca, 1991; Dorseuil, 1992; Kim, 1993; Eray, 1998) and antigen presentation (Patel, 1993; Weiser, 1994; Knight, 1997), there have been very few studies to directly assess the functional role of isotype-switched receptors.

With the results presented in this thesis as well as the elegant studies of Kaisho *et al.* (Kaisho, 1997) and Achatz *et al.* (Achatz, 1997), two complementary experimental systems have now been used to isolate the role of isotype-switched IgG BCRs in B cell responses to antigen *in vivo*. These studies can now be compared and contrasted to reveal the relative strengths and weaknesses of each.

In the first approach, gene targeting technology was used to generate knockouts of membrane-bound IgG_1 (Kaisho, 1997) and IgE (Achatz, 1997), leading to profound defects in IgG_1 and IgE production in the knockout mice. This showed very convincingly that membrane expression of IgG and IgE were critical for the normal function of B cells expressing those isotypes. As well, tail-truncation mutants were generated lacking the putative extended cytoplasmic sequences of IgG_1 and IgE, which were replaced by the 3 aa sequence common to membrane-bound IgM and IgD (see Figure 1-3). These were constructed to investigate the role of the highly conserved extended cytoplasmic tail sequences of IgG_1 and IgE. Interestingly, these tail-truncation mutant mice showed an intermediate defect in IgG_1 and IgE responses, suggesting a critical function for the cytoplasmic tail domain. In the case of IgG_1, Kaisho and colleagues showed that many fewer IgG^+ antigen-specific cells accumulated at the peak of the anti-NP response in the tail-truncation animals, and they also showed profoundly decreased serum IgG_1 titres and affinity maturation (Kaisho, 1997). This study concluded that the cytoplasmic tail sequences of IgG_1 were critical for the function of IgG^+ B cells, and it was also speculated on the basis of several *in vitro* observations (Weiser, 1994; Knight, 1997) that the IgG_1 cytoplasmic tail YXXM sequence might act as an antigen internalisation motif driving rapid B cell activation by the recruitment of T cell help.

The system outlined in this thesis took a complementary approach to the studies outlined above. To isolate the role of BCR isotype, this study used a panel of Ig transgenic

mice sharing the same high-affinity antigen receptors but expressing different "naïve" (IgM, IgD) or "memory" (IgG) isotypes. The use of adoptive transfers allowed affinity, isotype and precursor frequency to remain constant. This is in contrast to the study of Kaisho *et al.*, in which clear differences in affinity maturation and precursor frequency after the primary response were apparent in comparisons of tail-truncation and wild-type mice (Kaisho, 1997). Also, all of the transgenic B cells in this study were naïve, removing the difficulty of interpreting the responses of antigen-experienced versus naïve cells. It was found that transgenic B cells bearing IgG made a more robust response to antigen *in vivo* compared to transgenic B cells bearing IgM or IgD, due to a protective effect from cell loss during clonal expansion. To isolate the role of the unique membrane tail domain of IgG, the study in this thesis performed the "add-back" experiment of grafting the complete membrane tail (extracellular spacer, transmembrane and cytoplasmic sequences) of IgG onto IgM to see if it conferred any novel functions. Chimeric IgM/G transgenic B cells showed the same robust response to antigen as IgG transgenic B cells, demonstrating that the IgG membrane tail was the molecular determinant of increased clonal expansion and AFC production in this system. This finding does not support the conclusions of Kaisho and colleagues regarding the cellular mechanism behind the action of the IgG_1 cyoplasmic tail sequences. In the system outlined in this thesis transgenic B cells bearing the IgG_1 membrane tail were not activated more quickly than IgM transgenic B cells. Rather than impacting on cell activation or cell division, the IgG membrane tail instead seems to protect B cells from loss during clonal expansion.

While each study had produced different interpretations for the cellular mechanism behind the action of the IgG membrane tail, the two experimental approaches seemed to reach very similar conclusions about the critical role of this unit in determining the response of IgG^+ B cells to antigen *in vivo*. In complementary findings, ablation of the cytoplasmic sequences of the membrane tail reduces IgG^+ B cell responses, and the addition of the entire membrane tail domain onto IgM produces a full IgG-like cellular response. The advantage of the Kaisho study is that it narrows down the loss-of-function effect in the tail-truncation mutants to the cytoplasmic tail sequences of IgG_1, while the work presented in this thesis has only localised the gain-of-function effect using IgM/G chimeric mice to the entire membrane domain of IgG_1. If it is assumed that the deletion of the IgG cytoplasmic tail sequence in the tail-truncation mutants disrupted the protective effect of the IgG membrane tail domain, then it may be that the cytoplasmic tail of IgG is the key section of the IgG membran tail that mediates this effect.

How confident can we be in merging these two approaches to conclude that the IgG cytoplasmic tail sequences are responsible for the protective effects of IgG expression outlined in this thesis? While this is an attractive hypothesis, several questions remain,

especially concerning the interpretation of the loss-of-function tail truncation mutants. The study of Kaisho *et al.* relied on the surface expression of mutant IgG molecules carrying a disrupted, evolutionarily conserved domain. The study showed that there was a lower IgG Δtail level on LPS/IL-4 stimulated cells compared to wild-type B cells, suggesting a problem in the cell surface expression of the mutant IgG molecule. In addition, there was a profoundly lower IgG serum level in the pre-immune tail-truncation mice, which could be interpreted as a problem in IgG production even before immunisation. Taken together, an alternative interpretation for that study is that the truncation of the IgG cytoplasmic tail sequence led to poor or unstable IgG membrane expression, with the consequence of a lower IgG immune response.

This highlights the difficulties in dissecting the IgG membrane tail domain by deletional strategies. By including the entire conserved membrane tail domain of IgG in the IgM/G chimeric construct, Pogue and Goodnow were able to ensure equivalent surface expression amongst bone marrow B cells in IgM/G and IgM transgenic lines (Pogue, 2000), demonstrating that the gain-of-function findings presented here are not clouded by the same issues as the deletional study. A limitation of the work outlined in this thesis, however, is that it cannot isolate the relevant, minimal sequences within the IgG membrane tail domain that mediate the protective effects described here. It remains possible that the extracellular membrane-proximal region of IgG_1, which is also highly conserved amongst the IgG subclasses (see Figure 1-3) may be the key section of the membrane tail mediating biological effects, perhaps through isotype-specific interactions with the Igαβ heterodimer. This is speculation that cannot be presently resolved, however, and in the future mutational studies designed to dissect the IgG membrane tail domain will need to ensure correct membrane expression of the mutant molecules. It may be a more useful strategy to study IgG BCR signalling at a biochemical level, so that rational, minor mutational changes that minimise the possibility of disrupting Ig surface expression can be made to test hypotheses about the key sequences within the IgG membrane tail that mediate biological function.

8.2 (d) Candidate signalling pathways for the protective effect of the IgG membrane tail

The hypothesis presented in this thesis is that signalling *via* the IgG membrane tail has a protective, anti-apoptotic effect that allows greater net clonal expansion during a TD immune response *in vivo*. This has striking parallels to the role of CD28 during the co-stimulation of T cells, which has been shown to be a critical positive regulator of the size of the T cell clonal burst (Gudmundsdottir, 1999). One of the targets of CD28 signalling is Bcl-xL, and the induction of this anti-apoptotic protein is hypothesised to reduce the rate of T cell death over successive divisions during clonal expansion (Sperling, 1996). CD28

signalling does not just act to decrease cell death, but also increases the production of IL-2 and the rate of cell division (Gudmundsdottir, 1999), an effect not noticed during these studies of transgenic B cells expressing the IgG membrane tail. An important future experiment will be to test the role of apoptosis in limiting clonal expansion by transgenic B cells that do not express the IgG membrane tail. This could be done by over-expressing an anti-apoptotic protein such as Bcl-2 in the IgM transgenic line, to determine whether this rescues the clonal expansion response. This would provide good evidence that the relative balance of pro-apoptotic and anti-apoptotic factors is affected during antigen-driven expansion by the expression of the IgG membrane tail.

What candidate signalling pathways might the IgG membrane tail activate in order to shift the balance towards cell survival during clonal expansion?

Firstly, this thesis has presented preliminary evidence for differences in early signalling events in transgenic B cells bearing the IgG membrane tail. In particular, a consistent observation in this and previous work (Pogue, 1996) is a decreased induction of tyrosine phosphorylation of intracellular proteins in B cells expressing IgG, despite normal calcium responses. In addition, this thesis has described possible differences in the partitioning of receptors carrying the IgG membrane tail into detergent-resistant lipid microdomains. It is tempting to speculate that the IgG antigen receptor is arranged differently in the membrane compared to IgM or IgD, and might associate more closely with signalling-rich areas of the cell, leading to qualitatively different signalling. It is not presently clear what segment of the IgG membrane tail might be responsible for these early signalling differences.

Downstream from the receptor, transcription factors of the NFκB family are candidate signalling molecules involved in the balance of survival and death in B cells that may be differentially regulated by the IgG membrane tail. The activation of transcription factors of the NFκB family plays an important role in the regulation of numerous genes in many cell types (Pahl, 1999), and in naïve B cells NFκB induction is part of the spectrum of biochemical responses normally initiated by BCR signalling. By contrast, tolerant B cells with crippled BCR signalling only induce a subset of transcription factor responses, which do not include NFκB (Healy, 1997). The poor mitogenic responses in tolerant B cells correlated with a failure to up-regulate the anti-apoptotic protein A1 (Glynne, 2000), which has been shown to be induced by NFκB in B cells and to be essential for protection against BCR-induced apoptosis *in vitro* (Grumont, 1999). As B cells bearing the IgG membrane tail are even more resistant to cell loss, it will be interesting to determine if this is due to increased signalling *via* NFκB family members.

A possible connection between the IgG BCR and increased NFκB signalling is also seen in the observation that even on the RAG1$^{-/-}$ background a much greater fraction of IgG and IgM/G splenic transgenic B cells have a marginal zone phenotype compared to the IgM transgenic line. This may be due to an increased basal level of signalling *via* the NFκB pathway by the IgG BCR in resting B cells, as NFκB signalling has been implicated directly and indirectly in the control of marginal zone B cell numbers. Marginal zone B cells are absent from NFkB p50$^{-/-}$ mice (Cariappa, 2000) and are present in increased numbers in mice that over-express BAFF (Mackay, 1999; Batten, 2000), a recently described NGF/TNF ligand family member that binds the BAFF receptor on B cells (Thompson, 2001), and which may signal *via* NFκB (Do, 2000). This suggests that marginal zone maintenance or recruitment could be a reflection of the quality or amount of NFκB signalling in B cells.

Section 8.3. A model of the role of BCR isotype during memory responses

How do the findings of this thesis integrate into the wider literature concerning the mechanisms and features of memory antibody responses? Because this system controlled for factors such as antigen experience by using naïve transgenic B cells that only differed in BCR isotype, we do not know the role of BCR isotype on genuine memory B cells, which also have other unique characteristics. Nevertheless, the effect of the IgG membrane tail on clonal expansion by naïve transgenic B cells was striking, and may well have an important effect on memory cells.

Traditionally, memory responses are thought to be due to the increased antigen-specific precursor frequency as well as the qualitiative changes that accompany memory cell formation. This section will present a model speculating that expression of an isotype-switched BCR may be critical component of the qualitative changes that allow memory cells to be more easily activated and produce greater magnitude immune responses.

8.3 (a) Quantitative and qualitative changes lead to the rapid induction of the memory response

The rapid production and efficient triggering of the memory antibody response is explained by two key observations. Antigen-specific B and T cell precursor frequency is increased in immune animals relative to the naïve repertoire. This is a direct prediction of the clonal selection theory (Burnet, 1959), and is often cited as being of fundamental importance for the rapid, high rise in antibody titre during the memory response (Ahmed,

1996). In addition, recent work has shown that there are qualitative as well as quantitative differences between the memory and naïve lymphocytes. These also seem to play a critical role in determining the rapid kinetics of the memory response.

Precursor frequency

There is no doubt that elevated frequencies of antigen-specific B (Hayakawa, 1987; Schittek, 1990; Ridderstad, 1996; Maruyama, 2000) and T cells (Flynn, 1998) can persist for long periods as the remnants of a primary immune response. Also, it has been observed that immunising Ig transgenic mice (Toellner, 1996), increasing the frequency of limiting T help by carrier-priming (Liu, 1991; Toellner, 1996) and transferring Ig (Adams, 1990; Erickson, 2000) or TCR transgenic cells (Freer, 1995; Rathmell, 1995), or both (Garside, 1998), increases the magnitude of an immune response by providing many more antigen-specific precursor cells.

Is precursor frequency then the primary factor in determining the magnitude and kinetics of the normal memory response? While it makes intuitive sense, there are several pieces of evidence to suggest that memory responses might not rely on a large pool of antigen-specific cells, but rather on the qualitatively different properties of antigen-specific memory cells. For example, when the secondary B cell response to NP was analysed at the clonal level, it was estimated that only 18 genetically distinct B cell precursors were involved (Blier, 1987). In addition, Ridderstad *et al.* showed that mice carrying the Btk^{xid} mutation, while producing 17-fold fewer NP-specific memory B cells still made normal secondary responses. One interpretation of this finding is that excess memory B cells are usually maintained in normal animals and not all are required for recall responses, at least up to 5 weeks after the primary response. It is tempting to speculate that the "excess" memory B cells provide a reservoir for continual low-level differentiation into plasma cells to maintain protective immunity. One caveat of the Ridderstad study is that only the $B220^+$ memory population was enumerated, so it remains possible that the Btk^{xid} mutation affected the relative production of $B220^+$ and $B220^-$ memory cells (McHeyzer-Williams, 2000; Driver, 2001).

The study outlined in this thesis also suggests that differences other than precursor frequency affect the outcome of B cell responses to antigen. Transfer of as few as 10^4 IgG transgenic B cells augmented the endogenous primary response of C57BL/6 mice to TEL, while as many as 10^6 IgM transgenic B cells failed to increase the production of anti-TEL IgM (see Figure 3-1). This implies that the switch in isotype from IgM to IgG has a stronger effect on cell activation during an immune response than simply increasing the antigen-specific B cell frequency. Probably the immune system is aided by an elevated

frequency of antigen-specific cells during a memory response, but does not absolutely require a vastly elevated frequency of those cells for effective memory. More important are the qualitative changes that accompany the memory state that lead to the rapid, vigorous activation, expansion and differentiation of memory cells.

Cell migration and location

Numerous qualitative changes in both memory B and memory $CD4^+$ T cells potentially lead to their rapid activation on re-encounter with specific antigen. For antigen-specific memory B cells, this is partly due to increased BCR affinity allowing more efficient antigen capture, especially early during an infection when soluble antigen might be scarce. Also, the location of memory B cells near antigen-draining sites allows intimate contact with blood, lymph and mucosal environments (Spencer, 1985; Liu, 1988; Liu, 1991; Liu, 1995). The rapid, specific mobilisation of antigen-specific memory B cells within hours from the marginal zone to the outer PALS in rodents (Liu, 1991; Toellner, 1996) is a good example of this principle in action.

In this migration, the activated memory B cell resembles a primed DC, rapidly homing to the site where antigen-specific T help can be engaged. This is a good analogy, as one striking difference between naïve resting B cells and memory B cells is their ability to act as APCs. This reduces the requirement for DC priming of T cell help, as memory B cells can directly present antigen in a stimulatory form to antigen-specific T cells, many of which will be memory T cells with lower activation and co-stimulatory requirements (Byrne, 1988; Rogers, 2000).

Pre-activated state

Studies in human memory B cells have demonstrated constitutive expression of co-stimulatory B7 molecules (B7.1, B7.2), the rapid upregulation of those molecules after activation and the ability to activate T cells (Liu, 1995; Bar-Or, 2001). This a strong reciprocal collaboration, as memory helper T cells clonally expand, differentiate and secrete cytokines, which may then induce rapid isotype switching (Toellner, 1996), cell division and terminal differentiation in memory B cells. The lag period between the encounter of antigen and the production of antibody is therefore much shorter during the memory response compared to the primary (Liu, 1991; Toellner, 1996), because of rapid activation and expansion of memory B and T cells due to their pre-primed state.

The rapid production of plasma cells or effector T cells during the memory response may also indicate a different rate of differentiation for activated memory cells. The concept

that memory cells are "biased" towards terminal differentiation is not a new one (Celada, 1971), and it has received experimental support recently from a number of sources. For example, memory B cells isolated from human tonsil generated more AFCs than naïve B cells *in vitro*, while undergoing a similar rate of cell division (Arpin, 1997). Also, memory T cells produce cytokine-secreting effector cells more rapidly (Rogers, 2000).

This simple concept of "bias" has become more complicated, however, with recent reports suggesting phenotypic heterogeneity in memory B and T cell subsets that correlates with effector production. For example, $CD45RO^+$ $CCR7^+$ "central memory" and $CD45RO^+$ $CCR7^-$ "effector memory" T cells differ in their proliferative and differentiation capacity (Sallusto, 1999), as do $B220^-$ and $B220^+$ memory B cell subsets in the mouse (McHeyzer-Williams, 2000) and the $CD27^+$ $CD80^+$ and $CD27^+$ $CD80^-$ memory B cell subsets from human blood (Bar-Or, 2001). It may be that some memory cells are more "biased" towards effector production than others. A subset of memory cells making rapid effector responses in addition to a subset of memory cells able to make strong proliferative bursts before differentiation may be an ideal way of providing immediate protective immunity as well as ensuring the production of large numbers of effector cells.

8.3 (b) IgG expression as a component determining the magnitude of the memory response

The rapid initiation of the memory response is due to increased antigen-specific precursor frequency, a reduced requirement for priming and the rapid differentiation of both memory B and T cells into effectors. A general feature of memory cells seems to be a "relaxation" of activation and expansion requirements compared to naïve cells, and it may be that memory cells are equipped to overcome many of the normal limiting factors that are in place during the primary response. For example, there is evidence that memory $CD8^+$ T cells are activated quicker than naïve $CD8^+$ T cells, require less antigen, and have a faster cycle time with less death, leading to greater expansion *in vivo* (Veiga-Fernandes, 2000).

In the case of antibody responses, memory B cell responses can be induced by smaller amounts of antigen provided in the absence of adjuvant (Liu, 1991; McHeyzer-Williams, 1991; Kaisho, 1997), by-passing the requirement for the activation of innate mechanisms and DCs. This may be an example of a relaxation of the stringent rules that govern the differentiation and clonal burst of memory B cells relative to naïve cells. One controlling factor limiting the production of plasma cells during the activation of naïve B cells is associated with the splenic stroma (Sze, 2000) and may correspond to the number of $CD11c^{hi}$ PDCs able to sustain extrafollicular foci (Garcia de Vinuesa, 1999). Given the consistent association of memory B cells with robust extrafollicular expansion (Liu, 1991;

Toellner, 1996), it is tempting to speculate that memory B cells are less dependent on the putative survival signals provided by the splenic stroma or PDCs. Indeed, this study has shown that transgenic B cells expressing the IgG membrane tail make a more robust response in extrafollicular foci compare to transgenic B cells expressing IgM or IgD. Perhaps the protective, anti-apoptotic effect provided by the IgG membrane tail is the mechanism that reduces the dependence of IgG-expressing memory B cells on extrinsic survival signals during plasmablast expansion to lead to the production of many more effector plasma cells.

The hypothesis presented here for the role of IgG BCR expression in memory antibody responses can be summarised in the following way. For memory B cells, one of the key regulators of the clonal expansion process may be the expression of IgG, which has been shown in this study to protect B cells from death during clonal expansion. We know that TD memory responses to many protein and hapten antigens are dominated by IgG (Bauer, 1961; Nossal, 1989), and that this antibody is produced by memory B cells that express IgG (Okomura, 1976; Hayakawa, 1987; McHeyzer-Williams, 1991; Toellner, 1996), or switch to IgG rapidly after activation (Toellner, 1996). The protective effect of IgG during clonal expansion, in combination with other intrinsic changes in memory B cells such as higher affinity BCR (Eisen, 1964), location at antigen-draining sites (Spencer, 1985; Liu, 1988; Liu, 1991; Liu, 1995), improved APC function (Liu, 1995; Bar-Or, 2001) and a bias towards plasma cell differentiation (Arpin, 1997; McHeyzer-Williams, 2000) synergise to provide rapid, robust clonal expansion and differentiation. This leads to the production of many antigen-specific plasma cells and high titres of antigen-specific IgG, which are the hallmarks of a TD memory response.

Section 8.4. Future directions

This study has demonstrated that BCR isotype can influence B cell responses to antigen during an immune response *in vivo*. The expression of the unique IgG membrane tail is sufficient to protect naïve transgenic B cells from attrition during clonal expansion *in vivo*. This is a novel cellular mechanism not predicted by previous studies (Kaisho, 1997), and may play a critical role in the heightened antibody production seen during a memory antibody response.

An important question arising from this is the relative importance of BCR isotype in driving the memory response, compared to the changes in affinity, activation requirements and location that also occur. It will be an interesting extension of this work to compare memory transgenic B cells present after a normal primary response that express IgG or IgM BCRs, but are unable to switch BCR isotype. Do the protective effects of the IgG

membrane tail still allow greater expansion and antibody production, or do the multiple other intrinsic changes in memory B cells allow robust memory responses even in the absence of the IgG membrane tail?

Another key unanswered question is the biochemical mechanism by which the IgG membrane tail mediates its protective effect. One candidate signalling pathway that may be regulated differently is the key transcription factor NFκB, which is accessible to biochemical analysis during short term stimulation *in vitro*. To gain an unbiased view of similarities and differences between IgM isotype and IgG isotype signalling, it may be more fruitful to perform global gene expression profiling using microarrays. This could be done on both acutely stimulated IgG and IgM B cells, as well as B cells sorted on days 3 and 4 of an immune response, where the survival ability of IgG and IgM transgenic B cells seems to diverge.

The general relevance of the protective phenomenon reported in this study is also of interest. This study has shown that the IgG membrane tail mediates a protective effect during an immune response to the protein antigen HEL. To address whether IgG membrane tail expression allows more robust responses to other model antigens, the heavy chain-only MG line could be compared to the heavy chain-only MD line. In these lines, an elevated frequency of HEL-binding B cells is layered onto a more diverse repertoire. Lysozyme immunisation could then be directly compared to immunisation with other model antigens. If IgG membrane tail expression in non-lysozyme specific B cells allows more robust responses to other antigens, it would provide good evidence that the IgG membrane tail can augment clonal expansion as a general phenomenon.

Also, the role of the unique membrane tail domains of the other IgG subclasses as well as IgE and IgA remain to be resolved. Interestingly, Achatz and colleagues reported a reduction in IgE responses when the cytoplasmic sequences of that isotype were deleted by gene targeting (Achatz, 1997). They speculated that the IgE cytoplasmic sequence (which also contains a YXXM motif) acts in a similar way to IgG_1 by allowing more efficient antigen presentation. This thesis presents an alternative mechanism for the action of the IgG membrane tail and it is interesting to speculate that the IgE membrane tail may have a similar protective effect on IgE^+ B cells. The extracellular spacer and cytoplasmic sequences of the membrane-bound IgG, IgE and IgA subclasses are sufficiently different from each other, however, that it is impossible to predict whether they will share common functions.

Chapter 9. Appendix

Section 9.1. News and Views Article

Memory needs no reminders

Stephen Martin and Chris Goodnow

Your body can 'remember' specific diseases and, decades later, protect you from reinfection. This principle has been known for centuries, but immunologists are still struggling with the cellular mechanisms that underlie its maintenance. Is this long-term immune 'memory' due to the formation of a special class of long-lived memory cells, or to occasional 'reminders' to the immune system from residual antigens — the invading agents that induced the response in the first place. On page 636 of this issue[1] Maruyama and colleagues describe their use of an elegant genetic system to address the question. They suggest that B cells, the class of immune cell that makes antibodies, can persist as memory cells for long periods of time after immunization even in the total absence of specific stimulation by antigen.

To explain immune memory, Burnet and Talmage[2] proposed that rare antigen-specific B-lymphocytes, which are precursors of antibody secreting cells, expand clonally when they encounter antigen. This clonal expansion results in an enlarged pool of antigen-specific B cells that produces a faster, larger response the next time the antigen is encountered — that is, they carry a memory of the original immunization. However, there was then a divergence of ideas about how the expanded pool of cells could persist, and this divide of opinion remains. One line of thought proposes that intermittent re-stimulation of the memory B-cell population by specific or cross-reactive antigen is required. In the late 1960s it was discovered that intact protein antigen could exist in lymphoid tissues for months after immunization, in the form of antigen–antibody complexes on so-called follicular dendritic cells[3,4]. These immune complexes were thought to be an antigen depot for the continual stimulation of clonally expanded B cells[5] and for helper T cells, which aid in priming the antigen-specific B cells to produce antibodies. In support of this view, cell-transfer experiments[6] showed that functional B-cell memory declines rapidly with a half-life of around 3 weeks in the absence of antigen.

The contrasting opinion holds that memory B cells represent a specialized state of long-lived quiescent cells that do not require further stimulation by antigen. Supporting evidence for this comes from the finding that B-cell memory can be maintained in mice that lack follicular dendritic cells[7] and in mice deprived of helper T cells[8]. This shows that maintenance of memory B cells may not need antigen depots or signals from helper T cells. Moreover, immunological memory also occurs in the cytotoxic T that destroy virus-infected cells. Here,too, memory has been shown to persist under experimental conditions in which antigen is effectively absent[9,10]. This antigen-independent persistence of memory T cells may be a general theme of immunity and so apply to B cells too.

In their study, Maruyama *et al.*[1] used an inducible genetic switch to generate memory B cells in the absence of their specific antigen. The experiments involved genetically modifying mice to carry two different antibody genes specific for two structurally unrelated antigens. Antibody gene expression caused about 4% of the normal B cells in these mice to express specificity for the first antigen, nitrophenyl. However, although they had increased in frequency compared with an unmanipulated mouse, the nitrophenyl-specific B cells were still 'naive', having never encountered antigen before. The antibody gene specific for the second antigen, phycoerythrin, was held in an inactive form within each nitrophenyl-specific B cell. These two antibody genes were flanked by loxP sites, which allowed the genes to be modified by an enzyme called Cre recombinase. The recombinase gene itself was under inducible con-trol — Cre recombinase was made only when the mice were injected with the inducer, type-1 interferon.

Maruyama *et al.* immunized the mice with nitrophenyl to generate nitrophenyl-specific memory B cells. Memory B cells differ from naive B cells in that they are the 'antigen-experienced' progeny of the original naive B cells and are thought to be responsible for immune memory. After 8 weeks, when a pool of nitrophenyl-specific memory B cells had been established, the mice were treated with interferon. This activated the Cre recombinase, which, in a small proportion of memory B cells, caused the irreversible loss of nitrophenyl specificity and a switch to phycoerythrin specificity (Fig. 1). The phycoerythrin-specific memory B cells were predicted to persist if antigen is not required for memory-cell maintenance, or to die if antigen is essential (Fig. 2). The upshot was that these cells were maintained just as well as the nitrophenyl-specific memory B cells for 15 weeks after treatment with interferon.

Do these results rule in favour of the antigen-independent camp? The answer depends on how quickly one would expect the phycoerythrin-specific memory cells to die off in the absence of antigen, if antigen is indeed required for memory-cell persistence. The rapid decline of memory in cell-transfer experiments[6] suggests that memory cells would die off

with a half-life of around 3 weeks. Maruyama and colleagues' results clearly show that phycoerythrin-specific memory cells persist for much longer than that. But the apparent contradiction between the two sets of findings[1,6] may have its roots in the unusual experimental design of the cell-transfer experiments — by sampling B cells from thoracic duct lymph, as opposed to sampling non-recirculating B cells from the spleen, the study may have measured the short lifespan of activated B cells rather than that of memory cells. The more relevant question for Maruyama *et al.* is whether or not the phycoerythrin-specific memory cells die off more slowly than the phycoerythrin-specific naive B cells in the absence of antigen. Naive B cells have been shown to have a wide variety of lifespans, with an average half-life extrapolated to be at least 6–12 weeks[11–13], with many cells lasting for much longer. Given the potentially long lifespan of naive phycoerythrin-specific cells, a clear answer to whether antigen is needed to maintain pools of memory B cells must await experiments that monitor the decay of both naive and memory phycoerythrin-specific cells over longer periods, say 6–12 months.

The new findings[1] raise a challenging question for immunologists. If memory B cells do not require persistent immunizing antigen to survive for months or years, what stops large numbers of memory cells accumulating over a lifetime of different infections?

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Figure 1. Does immune memory need reminders from antigen? This is the genetic switch used by Maruyama et al.[1] to alter the specificity of memory B cells by genetic recombination. The antigens involved were nitrophenyl (NP) and phycoerythrin (PE). After the induction of nitrophenyl-specific memory B cells, treatment with interferon produced some phycoerythrin-specific memory B cells in the absence of their specific antigen. The lifespans of the nitrophenyl- and phycoerythrin-specific memory B cells were then compared.

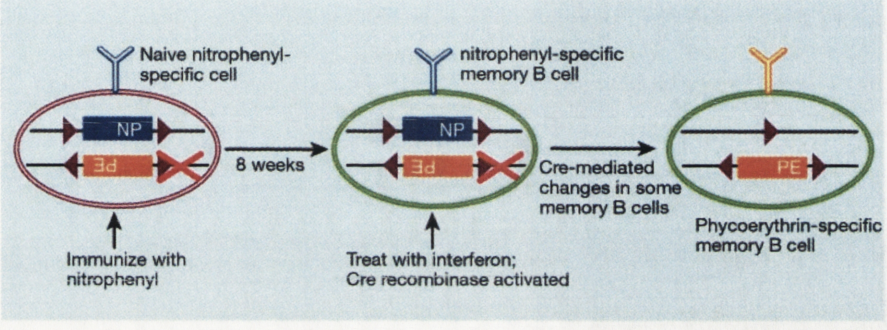
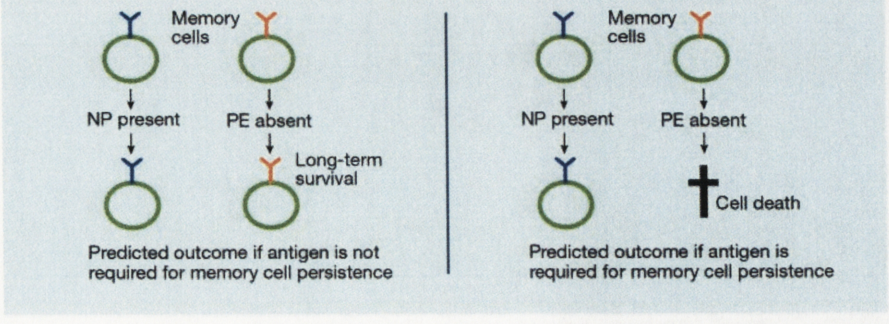


Figure 2. The predicted experimental outcomes of Maruyama and colleagues experiments[1]. The only antigen used in this experiment was nitrophenyl; phycoerythrin was completely absent. It follows that if specific antigen is required for the maintenance of memory B cells, the phycoerythrin-specific memory B cells will die; if specific antigen is unnecessary for persistence, these cells will survive. The authors found that the phycoerythrin-specific memory B cells persist just as well as the nitrophenyl-specific cells, and conclude that specific antigen is not required for the maintenance of memory B cells.



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